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Identification of diagnostic, prognostic and new major and minor susceptibility genes to pheochromocytoma and paragangliomas (PCC/PGL)

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Identification of diagnostic, prognostic and new major and minor susceptibility genes to pheochromocytoma and paragangliomas (PCC/PGL)

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ABSTRACT

BACKGROUND: Genetic diagnosis is recommended for all pheochromocytoma (PCC) and paraganglioma (PGL) cases (PPGL), as 65-80% are explained by a driver mutation in one of the 34 genes described so far. Several genetic testing algorithms have been proposed, but they usually exclude sporadic-PPGLs (S-PPGLs) and none include somatic testing. Moreover, as the list of PPGL related genes expands yearly, genetic diagnosis becomes a time-consuming task, and targeted gene panels using next generation sequencing (Targeted-NGS) have emerged as cost-effective tools.

AIMS: We aimed to elucidate the genetic heterogeneity of PPGL development through a systematic genetic study. This study was carried out in two consecutive parts.

MATERIAL AND METHODS: Part I included 329 probands and was focused on the genetic characterization of S-PPGL using Sanger sequencing (SS), and gross deletions of PPGL genes in which the mutational mechanism is relevant. Ninety-nine tumors from patients negative for germline mutations (GM) were available and tested for somatic mutations (SM) in *RET, VHL, HRAS, EPAS1, MAX* and *SDHB*. Part II addressed a blind genetic screening of PPGL based on 2 customized targeted-NGS assays. One of these panels allowed the study in germline and frozen tumor DNA, and the second one was specifically designed for DNA extracted from FFPE tissue. This second study included 453 PPGL patients (30 of them controls with known pathogenic mutations, and 275 had been partially screened by SS (WT^{PS})).

RESULTS: Part I: GM were found in 46 (14%) patients, being more prevalent in PGLs (28.7%) than in PCCs (4.5%) ($p=6.62\times10^{-10}$). Head and neck PGLs (HN-PGLs) and thoracic-PGLs (T-PGLs), more commonly had GMs ($p=2.0\times10^{-4}$ and p=0.027, respectively), but not abdominal-PGLs (A-PGLs). SM were found in 43% of those tested, being more prevalent in PCCs (48.5%) than in PGLs (32.3%) (p=0.13). Five metastatic cases and a quarter of S-PPGLs had a SM, regardless of age at onset. Part II: NGS assay sensitivity was ≥99.4%, regardless of DNA source. We identified 45 variants of unknown significance and 89 mutations, GMs in 29 (7.2%), and SMs in 58 (31.7%) of the 183 tumors studied (being 37 mutations found in WT^{PS}).

CONCLUSIONS: We recommend prioritizing testing of GM in patients with single HN-PGLs and T-PGLs, and for SM in those with single PCC. Catecholamine phenotype and SDHB-IHC should guide genetic screening, mainly in A-PGLs. Pediatric and metastatic cases should not be excluded from somatic screening. Both targeted-NGS assays are an efficient and accurate alternative to SS, facilitating the study of "minor" PPGL genes, and enabling genetic diagnoses in patients with incongruent or missing clinical data, that would otherwise be missed.

RESUMEN

ANTECEDENTES: El diagnóstico genético se recomienda en todos los pacientes con feocromocitoma (FEO) y paraganglioma (PGL), (FPGL), ya que el 65-80% se explican por una mutación en uno de los 34 genes descritos. Se han propuesto distintos algoritmos de diagnóstico genético, pero suelen excluir los FPGL esporádicos (FPGL-E) y ninguno incluye el estudio de mutaciones somáticas (MS). Además, como la lista de genes relacionados con FPGL no para de crecer cada año, el diagnóstico genético implica cada vez más tiempo, y los paneles de genes mediante secuenciación masiva (PG-NGS) emergen como una herramienta rentable y efectiva.

OBJETIVOS: Nuestro objetivo fue aclarar la heterogeneidad genética en el desarrollo de los FPGL mediante el estudio genético sistemático. El estudio se realizó en dos partes sucesivas.

MATERIAL Y MÉTODOS: La parte I incluyó 329 propósitus y se centró en la caracterización genética de pacientes con FPGL-E mediante la secuenciación por Sanger (SS) y las grandes deleciones de los principales genes relacionados con FPGL. Noventa y nueve tumores de los pacientes sin mutación germinal (MG) se incluyeron en el estudio de MS en *RET, VHL, HRAS, EPAS1, MAX* y *SDHB*. En la parte II el estudio genético se realizó de forma "ciega" utilizando 2 PG-NGS. Uno permitía el estudio en ADN germinal y de tumor congelado y el segundo fue específicamente diseñado para DNA extraído de tumor parafinado. En el segundo estudio se incluyeron 453 pacientes con FPGL (30 de ellos controles con mutaciones patogénicas conocidas y 275 habían sido parcialmente estudiados mediante SS (WT^{PS})).

RESULTADOS: Parte I: se encontraron MGs en 46 pacientes (14%), siendo más frecuentes en PGLs (28.7%) que en FEOs (4,5%) ($p=6.62\times10^{-10}$). Los PGLs de cabeza y cuello (CC-PGLs) y los torácicos (T-PGLs), más comúnmente presentaban MGs ($p=2.0\times10^{-4}$ y p=0.027, respectivamente), pero no los abdominales (A-PGLs). Se encontraron MSs en el 43% de los tumores estudiados, y fueron más frecuentes en FEOs (48,5%) que en PGLs (32.3%) (p=0.13). Cinco casos metastásicos y un cuarto de los FPGL-E presentaban una MS, independientemente de la edad. Parte II: el abordaje con NGS mostró una sensibilidad ≥99.4%, independientemente del tipo de ADN. Se identificaron 45 variantes de significado desconocido y 89 mutaciones, siendo MGs 29 (7,2%) y MSs 58 (31,7%) en los 183 tumores estudiados (37 se encontraron en los casos WT^{PS}).

CONCLUSIONES: Recomendamos priorizar el estudio de MG en los pacientes con un único CC-PGL y T PGL, y de MS en FEO. El fenotipo catecolaminérgico y la IHC-SDHB deberían guiar el estudio genético, principalmente en A-PGLs únicos. Los casos pediátricos y metastásicos no deberían excluirse del estudio somático. Ambos PG-NGS son una alternativa eficiente y precisa a la SS, que facilita el estudio de genes "minoritarios" de FPGL y el diagnóstico genético en pacientes con datos clínicos incongruentes o ausentes, que de otra manera no serían diagnosticados.

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ABBREVIATIONS

2SC - S-(2-Succinyl)cysteine

3-MT - 3-methoxytyramine

5-hmC - 5-hydroxymethylcytosine

11C-HED - 11C-hydroxyephedrine

18F-FDA - 18F-fluorodopamine

18F-FDG - [18F]-fluoro-2-deoxy-D-glucose

A-PGL - Abdominal paraganglioma

ATRX - Alpha thalassemia mental retardation X-linked

BAP1 - BRCA1 associated protein-1

BRAF - B-Raf proto-oncogene

CgA - Chromogranin A

CI - Confidence interval

CIMP -CpG island methylator phenotype

CNA - Copy number alteration

COSMIC - Catalogue of Somatic Mutations in Cancer

CSS - Carney-Stratakis syndrome

CT - Computed tomography

CTd - Carney triad

CVD - Cyclophosphamide, vincristine and dacarbazine

dbSNP - The Single Nucleotide Polymorphism database

DOPA - Dihydroxyphenylalanine

EGLN1 - egl-9 family hypoxia-inducible factor 1

EGLN2 - egl-9 family hypoxia-inducible factor 2

EPAS1 - Endothelial PAS domain-containing protein 1

ExAC - Exome Aggregation Consortium

EZH2 - Enhancer of zeste homolog 2

FFPE - Formalin-fixed paraffin embedded

FGFR1 - Fibroblast growth factor receptor 1

FH - Fumarate hydratase

GIST - Gastrointestinal stromal tumor

H3F3A - H3 histone, family 3A

HD - Hirschsprung's disease

HIF - Hypoxia-inducible factor

HN-PGL - Head and neck paraganglioma

ID - Identification

IDH1 - Isocitrate dehydrogenase type 1

IHC - Immunohistochemistry

INDELs - Small insertions and deletions

IQR - Interquartile range

JMJD1C - Jumonji domain containing 1C

KDM2B - Lysine (K)-specific demethylase 2B

KIF1B - Kinesin family member 1B

KMT2D - Lysine (K)-specific methyltransferase 2D

LOH - Loss of heterozygosity

LOVD - Leiden Open source Variation Database

MAX - MYC associated factor X

MDH2 - Malate dehydrogenase type 2

MEN - Multiple endocrine neoplasia

MERTK - Mer proto-oncogene tyrosine kinase

MET - Met proto-oncogene

MIBG - Metaiodobenzylguanidine

miRNA - MicroRNA

MITF - Microphthalmia-associated transcription factor

MRI - Magnetic resonance imaging

MTC - Medullary thyroid carcinoma

mTOR - Mechanistic target of rapamycin

NET - Neuroendocrine tumor

NF1 - Neurofibromatosis type 1

NGS - Next Generation Sequencing

PASS - Pheochromocytoma of the Adrenal gland Scales Score

PCC - Pheochromocytoma

PET - Positron emission tomography

PGL - Paraganglioma

PHD - Prolyl hydroxylase domain

PHP - Primary hyperparathyroidism

PNMT - Phenylethanolamine N-methyltransferase

PolyPhen-2 - Polymorphism Phenotyping v2

PPGL - Pheochromocytomas and paragangliomas

HRAS - Harvey rat sarcoma viral oncogene homolog

RET - Ret proto-oncogene

RTK - Tyrosine kinase receptor

S - Sensitivity

SDH - Succinate dehydrogenase

SETD2 - SET domain containing 2

SIFT - Sorting Intolerant From Tolerant

SNP - Single nucleotide polymorphism

SPECT - Single-photon emission computed tomography

S-PPGL - Sporadic PPGL

SSTR - Somatostatin receptor

TA-PGL - Thoracic-abdominal paraganglioma

TCA - Tricarboxylic acid

TCGA - The Cancer Genome Atlas

TERT - Telomerase reverse transcriptase

TGP - Targeted gene panel

TMEM127 - Transmembrane protein 127

TP53 - Tumor protein p53

T-PGL - Thoracic paraganglioma

UMD-VHL - The Universal Mutations Database for VHL mutations

VHL - von Hippel-Lindau

VUS - Variant of unknown significance

WES - Whole-exome sequencing

WT - Wild type, no known genetic mutation

RECIST - Response Evaluation Criteria In Solid Tumors

I. INTRODUCTION

1.1 DISEASE DEFINITION AND ANATOMY

Pheochromocytomas (PCCs) and paragangliomas (PGLs), together referred as PPGL, are neuroendocrine tumors (NETs) derived from the chromaffin cells of the embryonic neural crest that develops into sympathetic and parasympathetic paraganglia. Neoplasias derived from sympathetic paraganglia tend to be catecholamine-secreting tumors and can be located either in the adrenal medulla (PCC) or at the thoracic (T-PGL) and/or abdominal (A-PGL) region, whereas tumors derived from parasympathetic paraganglia are mainly non-secreting tumors mostly located in the head and neck area (HN-PGLs), and in minor percentage in the thorax[1].

Thoracic-abdominal PGLs (TA-PGLs) most commonly arise around the inferior mesenteric artery (the organ of Zuckerkandl), the aortic bifurcation, and less frequently in chest and pelvis. HN-PGLs arise preferentially from vascular regions (the jugular bulb, and the carotid body) or along the glossopharyngeal and/or the vagus nerves[2–5] (**Figure 1**).

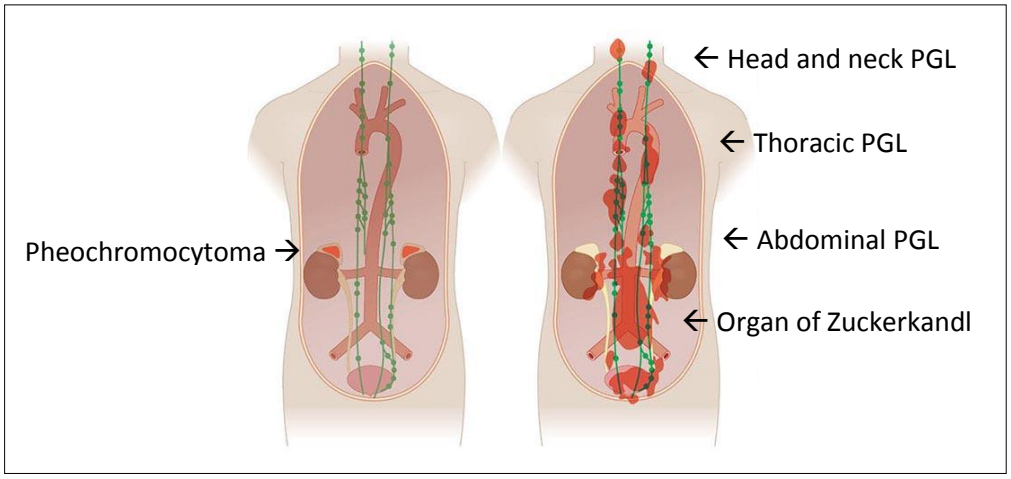


Figure 1. Location of PPGL. Normal paraganglia is colored in green, and tumors in orange.

1.2 EPIDEMIOLOGY

The prevalence of PPGL has been estimated to be between 1:4500 and 1:1700[3], being the prevalence in patients with arterial hypertension 0.2-0.6% (1.7% in children). Up to 20% of PPGL are diagnosed during childhood[6], being PCC the most frequently diagnosed endocrine tumor in children[7]. Diagnosis of PPGL may be missed during life, as PCC are diagnosed as incidentally discovered adrenal masses during imaging studies for other reasons in 5% of patients, and autopsy studies have demonstrated undiagnosed tumors in 0.05-0.1%[8]. Annual incidences of PPGL (cases per million) in the general population[3] and in children[6] are 3–8 and 0.3, respectively. The only statistics in Spanish population dates from 1994 and reported an incidence of 2.06 in the South of Galicia[9].

PPGL can occur at any age, but the peak incidence occurs in the third to fifth decades of life. The average age at first PPGL diagnosis is 24 years in hereditary cases and 43 years in sporadic cases[1], with an equal incidence between males and females, except under the age of 10 in which there is a slight predominance in males[3,6]. The only environmental risk factor described is chronic hypoxia, which, in populations living at high altitude, leads to an increased incidence of HN-PGLs[1,6]. Combining two large series of 693 unselected PPGL patients the type of tumor was PCC in 69%, TA-PGL in 15%, and HN-PGLs in 22% (some patients having combinations of tumors)[5,10,11].

1.3 PROGNOSIS

The metastases rate of PPGL ranges from less than 1 % to more than 60 %, depending on tumor location, size and genetic background[2]. Although features such as size (larger than 5 cm), extraadrenal location of primary tumors[5,12], a high "Pheochromocytoma of the Adrenal gland Scales Score" (PASS), or increases in plasma 3-methoxytyramine (3-MT, a dopamine-DOPA metabolite)[13,14] provide useful information to assess the likelihood of metastatic disease, the finding of mutations in *SDHB* is the only criterion strongly associated with an increased risk of metastases at diagnosis or during follow-up: 30% (range 20-70)[4,15–17]. However, for patients with apparently benign primary tumors, the mean incidence of metastatic recurrences and new tumors during follow-up is 11.3 % and 6.2%, respectively, being those patients harboring a germline mutation the ones with a higher probability of both[18]. Prognosis of metastatic PPGL is poor, with a 5-year mortality rate greater than 50%[19,20].

Nowadays metastatic PPGL remain a diagnostic challenge, as currently there are no reliable cytological, histological, immunohistochemical, or molecular criteria for malignancy[21], and the diagnosis remains strictly based on the finding of metastases where chromaffin cells are not usually present[22]. Metastases have been reported to be located in lymph nodes in around 80%, bones in 71%, and lungs and liver in 50% of metastatic cases[4,22–24]. The diagnosis is usually obtained from imaging studies, as histological confirmation is rarely available[25]. Consequently, metastases in PPGL can only be defined in advanced stages, and the inability to predict tumor behavior does not allow an optimal therapeutic planning[24].

Recently, different studies have attempted to predict metastatic potential through different measurements such as the presence of tumor necrosis, high Ki-67 index (>4%)/mitotic count, or pS100 absence[26] in pathological study, overexpression of HIF-α and its target genes[27,28], extremely high mRNA copy numbers of a variant of carboxypeptidase E[29], overexpression of the microRNA (miRNA) 183 (miR-183) in tumors[30,31], or the hypermethylation of the negative

elongation factor complex member E gene[32] among others, but further studies are needed to confirm the predictive value of these markers, especially during diagnosis procedures.

1.4 PPGL-ASSOCIATED SYNDROMES

PPGL can develop in an apparently sporadic presentation, or as part of several tumor syndromes associated with alterations in distinct genes. While initially it was thought that only 10% of cases were caused by germline mutations, after discovering an increasing list of PPGL-related genes, nowadays PPGL show the highest degree of heritability of all human tumors[33]. Thus, currently it is recognized that a genetic germline mutation explain at least 40% of patients, including cases with features suggesting inheritability (such as early age at onset, multiple and/or metastatic tumors and/or family history of PPGL or other syndrome-associated tumors), and 8-12% of apparently sporadic PPGL[11,33–40]. In pediatric cases up to 70-80% harbor a germline mutation, regardless of their family history[41,42].

Approximately 40% of PPGL develop primarily in the context of three familial tumor syndromes: von Hippel-Lindau disease (VHL) caused by *VHL* mutations, multiple endocrine neoplasia type 2 (MEN2) caused by *RET* mutations, and familial PPGL: 1) hereditary PGLs, caused by mutations in succinate dehydrogenase (*SDH*), fumarate hydratase *(FH)* and malate dehydrogenase type 2 (*MDH2)* genes*;* and 2) familial PCCs, caused by mutations in the transmembrane protein 127 (*TMEM127)* or the MYC associated factor X (*MAX*) genes. A small fraction of PPGL are associated with other syndromes: the Carney triad (CTd) defined by the coexistence of PGL, gastrointestinal stromal tumor (GIST), plus pulmonary chondroma, and the Carney-Stratakis syndrome (CSS) characterized by PGL and GIST[43]. Both CTd and CSS have been related to *SDH* genes mutations, but whereas CSS is almost always caused by mutations in *SDH* genes, they appear rarely in CTd. However, epigenetic *SDHC* promoter mutations have been recently linked to CTd[43–45]. The presence of PPGL in two syndromes classically related with PPGL, multiple endocrine neoplasia type 1 (MEN1) and neurofibromatosis type 1 (NF1), has been finally found to be rare: <1%[5] and 0.1-5.7%[46], respectively. Latterly, two additional syndromes have been linked to PPGL: the Pacak-Zhuang syndrome and syndromes associated with leiomyomatosis, being related to mutations in the endothelial PAS domain-containing protein 1 *EPAS1/HIF2A* (*EPAS1*) gene[47] and *FH*[48], respectively. To note, each syndrome presents a set of signs and tumors with overlap between them, and they are detailed in **Table 1**.

Hereditary cases mainly follow an autosomal dominant mode of transmission. Exceptions to this rule are the inheritance linked to *SDHD*[49], *SDHAF2/SDH5 (SDHAF2)*[50] and *MAX*[51]mutations. In these cases, only those carriers that inherit the mutation from their fathers will develop the

Table 1. Summary of phenotypic and genetic features associated with the described PPGL related genes.

Gene	Driver or 2nd hit	Chr. Location	Type of gene	Cluster	Inheritance	Mean age	Germ.	Som.	Mos.	GD	Risk of malignancy	Predominant tumor location	Number of tumors	BC	Related syndrome Associated tumors/features
FH	Driver	1q42.1	TSG	C1A	(AD)	NR	<1-5 (0.8%)	<1 (1)	NR	Yes[52]	High (60%)[53]	PCC+TA>HN	Multiple	(NA)	PGL8; Reed syndrome or Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC); multiple cutaneous and uterine leiomyomatosis (MCUL); cutaneous and uterine leiomyomas; type 2 papillary renal carcinoma[54]
IDH1	Driver	2q34	TSG	C1A	?	NR	NR	<1 (1)	NR	NR	?	HN	Single	(NA)	None reported [55]
MDH2	Driver	7q11.23	TSG	C1A	(AD)	NR	<1% (1)	NR	NR	NR	?	TA	Multiple	(NA)	Early-Onset Severe Encephalopathy[56]
SDHA	Driver	5p15.33	TSG	C1A	(AD)	40	<1-5	<1 (1)	NR	NR	Mod. (<10%)	TA>>PCC	Single	(NA)	PGL6; Leigh syndrome (homozygous patients, but no PPGL described); CCRC; GIST; pituitary adenoma.
SDHAF2 /SDH5	Driver	11q12.2	TSG	C1A	AD, paternal	30-40	<0.1-1	0	NR	NR	Low	HN>>PCC	Multiple (87%)[5]	(NA)	PGL2
SDHB	Driver	1p36.13	TSG	C1A	AD	30	10	<1	NR	Yes	High (30-70%)	TA>HN>PCC	Multiple (21%)[5]	NA, DA	Carney-Stratakis syndrome; PGL4; CCRC; GIST; pituitary adenoma; thyroid carcinoma.
SDHC	Driver	1q23.3	TSG	C1A	AD	40-50	<1-5	0	Yes	NR	Low	HN>TA>PCC	Multiple (17%)[5]	(NA)	Carney-Stratakis syndrome; PGL3; CCRC; GIST; pituitary adenoma.
SDHD	Driver	11q23.1	TSG	C1A	AD, paternal	35	9-10	<1	NR	NR	Low (<5%)	HN>TA>PCC	Multiple (56%)[5]	NA, DA	Carney-Stratakis syndrome; PGL1; renal cell carcinoma; GIST; pituitary adenoma; thyroid carcinoma; NET (?)[57]
EGLN1/ PHD2	Driver	1q42.1	TSG	C1B	?	NR	<1 (2)	NR	NR	NR	?	TA>PCC	Multiple	(NA)	Hereditary polycythemia; polycythemia[58].
EGLN2/ PHD1	Driver	19q13.2	TSG	C1B	?	NR	<1 (1)	NR	NR	NR	?	TA>PCC	Multiple	(NA)	Hereditary polycythemia; polycythemia[58].
EPAS1/ HIF2A	Driver	2p21	O	C1B	?	NR	<1-5 (1)	5-7	Yes	NR	?	TA>PCC	Multiple	NA	Familial erythrocytosis type 4; Pacak-Zhuang; polycythemia; somatostatinoma.
VHL	Driver	3p25.3	TSG	C1B	AD	30	7-10	10	Yes	Yes	Low (<5%)	PCC (Bil PCC 50%)>>>TA, HN 30-55% PPGL as the first manifestation of VHL	Multiple	NA> DA	von Hippel Lindau (I 1/36 000): 10-25% present PPGL CCRC, hemangioblastomas of CNS/retina/kidney and pancreas, pancreatic NET and cysts, endolymphatic sac tumors of the middle ear, papillary cystadenomas of the epididymis and/or broad ligament. Autosomal recessive congenital polycythemia (also known as familial erythrocytosis type 2.
ATRX	Driver and 2nd hit	Xq21.1	TSG	C2A	?	NR	NR	12.6	NR	NR	?	PCC, PGL	(Single)	?	X-linked alpha thalassemia mental retardation syndrome (germline mutation); gliomas, neuroblastomas, medulloblastomas and NET (?).
HRAS	Driver	11p15.5	O	C2A	?	NR	NR	10	NR	NR	Low	PCC>PGL	Single	(A)	Costello syndrome (germline).
H3F3A	?	1q42.12	O	C2A	?	NR	NR	NR	Yes (7%)	NR	?	PCC, A-PGL	?	(A)	Giant cell carcinoma of bone (?); Glioma (?).
KIF1B	Driver	1p36.22	TSG	C2A	(AD)	NR	<1 (2)	<1 (2)	NR	NR	?	PCC (Bil?)	?	(A)	Neuroblastoma (?), ganglioneuroma (?), leiomyosarcoma (?); Lung adenocarcinoma (?); Colorectal carcinoma (?)[59].
MAX	Driver	14q23.3	TSG	C2A	AD, paternal	32	<1-5 (1.1%)	<5	NR	Yes[60]	Mod. (10%)	PCC (Bil PCC 68%)>PGL	Single	A> NA	PGL7/FPCC2; renal oncocytoma (?)[60].

Gene	Driver or 2nd hit	Chr. Location	Type of gene	Cluster	Inheritance	Mean age	Germ.	Som.	Mos.	GD	Risk of malignancy	Predominant tumor location	Number of tumors	BC	Related syndrome Associated tumors/features
NF1	Driver	17q11.2	TSG	**C2A**	AD	42	<3-5	20-40	Yes	Yes	Mod. (12%)	PCC 95% (Bil PCC 16%)>TA	Single	A +NA	von Recklinghausen's disease (I 1 : 2500–3000): 0.1-5.7% present PPGL, 3.3-13% based on autopsy studies[35]. Café-au-lait spots, neurofibromas, axillary and inguinal freckling, Lisch nodules (iris hamartomas), bony abnormalities, optic/CNS gliomas, malignant peripheral nerve sheath tumors, macrocephaly, and cognitive defects.
RET	Driver	10q11.21	O	**C2A**	AD	30-40	5-10	10	NR	NR	Low (<5%)	PCC (Bil PCC 50-80%)>>>TA, HN 12-25% PPGL as the first manifestation of MEN2[5]	Multiple	A +NA	MEN2 (I 1/30000-40000): 50% present PPGL Medullary thyroid carcinoma (95% MEN2A, 100% MEN2B). Parathyroid adenomas (15-30%), notalgia or cutaneous lichen amyloidosis, Hirschsprung disease (MEN2A or Sipple syndrome) Marfanoid habitus, mucocutaneous neuromas, myelinated corneal nerves, gastrointestinal ganglioneuromatosis (MEN2B, MEN3 or Gorlin syndrome).
TMEM127	Driver	2q11.2	TSG	**C2A**	AD	43	<1-5 (0.9%)	0	NR	NR	Low (<5%)	PCC (Bil PCC 33-39%)> TA, HN	Single	A +NA	PGL5/FPCC1; renal cell carcinoma (?).
MET	Driver and 2nd hit	7q31	O	C2B	?	NR	<1 (1)	2.5 (5)	NR	NR	?	PCC	?	(A)	Papillary renal cancer[30,61].
BAP1	?	3p21.1	TSG	?	(AD)	NR	<1 (1)	NR	NR	NR	?	PGL	?	?	Uveal/cutaneous melanoma; mesothelioma; CCRC (?)[62].
BRAF	?	7q34	O	? (C2?)	?	NR	NR	1,2 (1)	NR	NR	?	PCC	(Single)	?	Melanoma (?); colorectal cancer (?).
EZH2	?	7q36.1	TSG	?	?	NR	2 (1)	NR	NR	NR	?	(PCC)	?	?	Lymphoma; myeloid malignancies.
FGFR1	?	8p11.23	O	? (C2?)	NR	NR	2 (1)	NR	NR	NR	?	PCC	(Single)	?	Glioblastoma.
JMJD1C	?	10q21.3	TSG	?	?	NR	?		NR	NR	?	(PCC)	?	?	
KDM2B	?	12q24.31	?	?	NR	NR	NR	2 (1)	NR	NR	?	(PGL)	?	?	
KMT2B/ MLL4	?	19q13.12	?	?	?	NR	2 (1)	NR	NR	NR	?	PGL	(Multiple)	?	
KMT2D/ MLL2	?	12q13.12	O	?	?	NR	(2)	(12)	NR	NR	?	PCC	(Single)	?	Kabuki syndrome; gliomas, neuroblastomas, medulloblastomas and NET[30,63].
MEN1	Driver	11q13	TSG	?	AD	NR	<1	NR	NR	Yes[64]	?	PCC	Single	?	MEN1 syndrome (I 1/30000) : <1% present PPGL. Primary hyperparathyroidism; pituitary adenoma; gastroenteropancreatic NET; adrenal cortical tumors, carcinoid tumors, facial angiofibromas, collagenomas, and lipomas.
MERTK	?	2q13	O	?	?	NR	2 (2)	NR	NR	NR	?	PCC, PGL	?	?	Medullary thyroid carcinoma (?).
MITF	?	3p13	O	?	AD	NR	NR	NR	NR	NR	?	PCC>> TA, HN	Single	?	Melanoma; renal cell carcinoma; pancreatic carcinoma[63,65].
SETD2	?	3p21.31	TSG	?	?	NR	2 (1)	NR	NR	NR	?	(PCC)	?	?	Renal cancer; leukemia.
***TERT* promoter**	?	5p15.33	O	?	?	NR	NR	11.1 (2)	NR	NR	?	A>PCC	Single	?	
TP53	?	17p13.1	TSG	?	?	NR	NR	2.35 (2)	NR	NR	?	PCC	(Single)	?	Li Fraumeni-like syndrome; adrenal cortical carcinoma, breast cancer, choroid plexus carcinoma, and osteosarcoma.

Chr: chromosome; ?: unknown; TSG: tumor suppressor gene; O: oncogene; (): it is not clear; AD: autosomal dominant; NR: not reported; Germ.: germline mutations - percentage (number of cases described); Som.: somatic mutations – percentage (number of cases described); Mos.: mosaicism; GD: gross deletions; Mod.: moderate; PGL: paraganglioma; PCC: pheochromocytoma; A: abdominal PGL; TA: thoracic-abdominal PGL; HN: head and neck PGL; Bil: Bilateral; BC: Biochemical predominant secretion; NA: noradrenergic (predominant secretion of noradrenaline/normetanephrine); A: adrenergic (predominant secretion of adrenaline/metanephrine); DA: dopaminergic (secretion of dopamine/3-methoxytyramine); I: incidence[66]; GIST: gastrointestinal stromal tumor; CNS: central nervous system; CCRC: clear cell renal carcinoma; NET: neuroendocrine tumor.

disease, although the underlying mechanism is not totally clear. Despite initially it was though that *SDHD* and *SDHAF2* presented maternal imprinting, exceptions of maternal transmission have been reported[67,68], and further research is needed to elucidate the real mechanism. In addition, an incomplete penetrance has been shown for *SDHA, SDHC*[69], *SDHB*[70], *TMEM127*[71], *FH*[53], and *MDH2*[72]. However, only data for *SDHB* have been reported, being 30% (95% confidence interval (CI) 17–41%) the average of the penetrance of tumors at age 80 of all *SDHB* carriers[70].

The genetic scenario of sporadic PPGL changed in 2011 when it was reported that 14% of PPGL could be explained by somatic mutations in *RET* and *VHL*[73]. One year later *NF1* was found to be somatically involved in an additional 24-41% of PPGL[74,75]. Other genes explaining heritable susceptibility have been also found to be somatically mutated (*SDHB*[76], *SDHD*[77], *SDHA* (TCGA data), *MAX*[78]); however their somatic involvement is scarce. In addition, new key players were discovered in the sporadic presentation, such as *HRAS*[79] and *EPAS1*[80]. Interestingly, *EPAS1* was firstly described to cause PPGL through somatic mosaicism[47,81], a mechanism that had been previously described at least for *NF1*[82] and *VHL*[83] mutations. Consequently, nowadays it is clear that somatic mutations play an important role in PPGL as they have been described in up to 40% of tumors[1,84].

1.5 ELUCIDATING THE GENETIC SCENARIO OF PPGL

The first genes with mutations described as cause of PPGL were those responsible of specific syndromes, such as NF1 (*NF1*), MEN2A (*RET*), VHL (*VHL*), and MEN1 (*MEN1*), as some patients affected by these diseases developed PPGL (especially PCCs). In 2000, targeted mutational analysis in families affected by HN-PGLs lead to the discovery of *SDHD*[49], a component of the succinate dehydrogenase mitochondrial complex II (SDH), being the first human tumor model found to carry an inherited mutation in a gene encoding a metabolic enzyme[33]. Later, the other members of the complex were found to be involved in PPGL pathogenesis as well: *SDHC*[85], *SDHB*[86], *SDHAF2*[50], and finally *SDHA*[87].

Combining data from gene expression profiles performed in 2004 by Eisenhofer et al.[88] and in 2005 by Dahia et al.[89] it was possible to know that tumors with mutation in *VHL*, *SDHB* and *SDHD* presented an overexpression of angiogenesis/hypoxia pathways related-genes (cluster 1), in comparison with *RET*- and *NF1*-tumors, which showed overexpression of genes related to the RAS/RAF/MAPK and PI3K/AKT/mTOR kinase signaling pathway (cluster 2). In addition, it was already established that cluster 1 tumors shared a noradrenergic secretion, while cluster 2 was enriched with tumors producing both adrenaline and noradrenaline. Further methylation studies showed that the noradrenergic secretory phenotype of cluster 1 tumors was caused by

low expression of phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine, through the hypermethylation of the *PNMT* promoter. Posterior studies performed by Favier et al.[90] and our group[91] distinguished two subclusters in cluster 1 based on the activation of distinct pseudo-hypoxic pathways, and finally, a DNA methylation profiling uncovered that one of these subtypes in cluster 1 showed an hypermethylator phenotype (cluster 1A)[48].

The use of Next Generation Sequencing (NGS) tools has been a key point to elucidate new players in the genetic scenario of PPGL. Due to the relatively high cost and the ethical concerns regarding incidental findings, whole-exome sequencing (WES) has been mainly used in research settings[48,51,72,79,92,93], while targeted gene panels (TGPs) have shown a greater applicability as a diagnostic tool, being faster, cheaper and more sensitive, even in cases with mosaicism[47,81–83], than the classically used Sanger sequencing[94–99]. In addition, TGPs enable the screening of genes systematically excluded in Sanger sequencing study due to their large size or rarity of their mutations, and facilitate patient selection for the screening of new genes, large rearrangements or the use of 'omic platforms (e.g. to detect mutations beyond coding regions)[30].

Using Sanger sequencing of a candidate region, and combining 'omic data with NGS and/or copy number alteration (CNA) data for tumors without known mutations attributed to cluster 1 or 2, new genes were discovered. *TMEM127*[100]*, MAX*[51]*,* and *HRAS*[79] were described as driver genes for cluster 2 tumors, and *FH*[48]*, EPAS1*[101]*,* and *MDH2*[72] for cluster 1. In addition, other genes have been described in the last years, but they seem to play a minor role in PPGL ("minor" genes) since the mutations have been described in isolated families (*KIF1B*, *BAP1, EGLN1/PHD2* (*EGLN1*)[33], and *EGLN2/PHD1* (*EGLN2*)[58]); in few sporadic cases (isocitrate dehydrogenase type 1 (*IDH1*)[55], *MERTK*, *H3F3A*, *SETD2*, *EZH2*, *FGFR1*[93] and *BRAF*[95]); or mainly reported in patients with mutations in recognized PPGL driver genes, suggesting a secondary role (*ATRX*[102], *TP53*[95], *JMJD1C*, *KDM2B*[93], *KMT2D/MLL2,* and *MET*[30]). Finally, germline *MITF* mutations[65] and mutations outside the exonic region have been recently described, such as promoter alterations in *TERT*[103] or epi-mutations in *SDHC*[104]*.* Some clinical features have been related to mutations in these genes, but the limited number of cases described needs further studies before establishing a real association (**Table 1**).

Despite this heterogenic genetic background, integrative genomic studies have provided evidence for strong concordance between genetic status and multi-omics data (transcriptomic gene expression, CNA, metabolomics signature, miRNA profiles and DNA methylation), allowing

to classify PPGL tumors into two main clusters and five molecular subgroups, each one displaying a specific set of genomic alterations and related clinical characteristics[30,31,35,51,84,105](**Figure 2**).

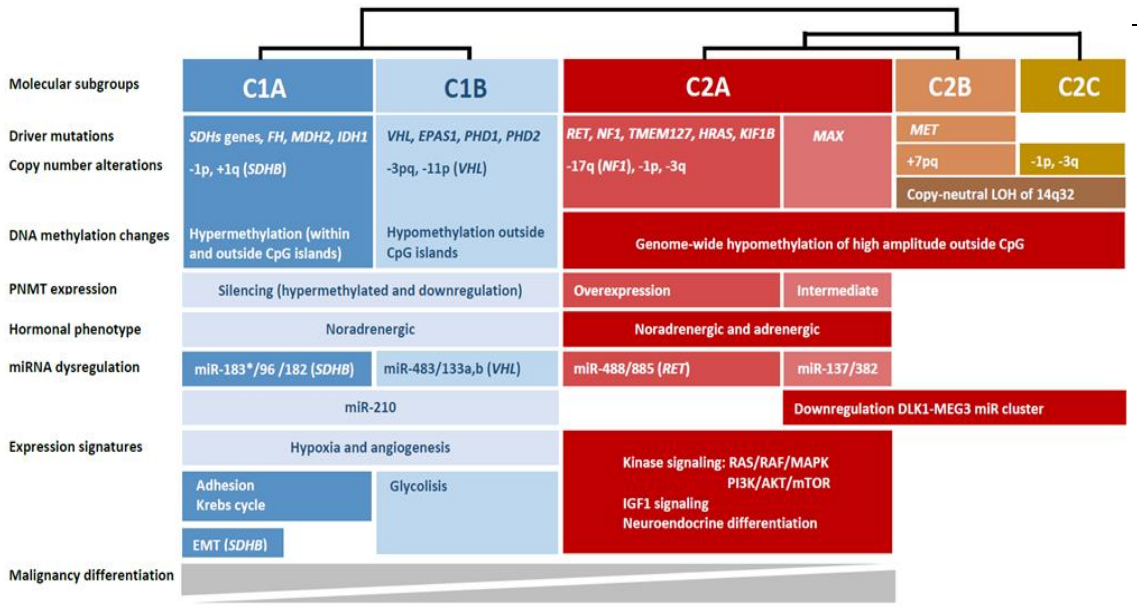


Figure 2. Molecular signatures of PPGL subtypes.
PNMT: phenylethanolamine N-methyltransferase; EMT: epithelial-to-mesenchymal transition; LOH: loss of heterozygosity. *Related to metastatic cases[31]. Adapted from[30,31,35,51,84,105].

1.5.1 CLUSTER 1: Pseudo-hypoxia cluster

Altered genes related to this cluster cause the so called pseudo-hypoxic response by stabilizing hypoxia-inducible factors (HIFs) under normoxic conditions[84].

Under normal oxygen tension, the degradation of α subunits of HIF (HIF1α, 2α, and 3α) is initiated through its hydroxylation by prolyl hydroxylase domain (PHD) proteins: PHD1, PHD2, and PHD3 (encoded by *EGLN2*, *EGLN1*, and *EGLN3* genes, respectively). Under normoxia conditions, PHDs use oxygen and α-ketoglutarate to hydroxylate HIF prolyl residues. The hydroxylated HIFα is then targeted by the von Hippel-Lindau protein (pVHL), a component of the E3 ubiquitin ligase complex, which modifies HIFs for their degradation in proteasomes. On the other hand, under hypoxia conditions, HIFα is stabilized and binds to the HIFβ subunit to form an active transcription factor that regulates expression of a large repertory of genes involved in angiogenesis, cell survival, polycythemia, and tumor progression.

· CLUSTER 1A: Krebs cycle cluster and familial PGLs

This subcluster is characterized by the Krebs cycle reprogramming and with oncometabolite accumulation or depletion. It contains tumors with mutations in *SDH* genes, *FH*, *MDH2,* and *IDH1.*

SDH genes encode SDH, a mitochondrial enzyme responsible for reactions in the tricarboxylic acid (TCA) cycle, where it catalyzes the oxidation of succinate to fumarate, and in the respiratory electron transfer chain (complex II of the mitochondrial respiratory chain), where it transfers electrons to coenzyme Q. SDH is a heterotetramer composed of four proteins: two catalytic (SDHA and SDHB), and two structural (SDHC and SDHD) that anchor the complex to the mitochondrial inner membrane. An associated protein, SDHAF2, is a highly conserved cofactor of flavin adenine dinucleotide which is implicated in the flavination of SDHA and is essential for SDH function[106]. Otherwise, *FH*, *MDH2,* and *IDH1* encode other TCA cycle enzymes involved in the reversible hydration/dehydration of fumarate to malate, the reversible conversion of malate to oxaloacetate with the concurrent reduction of NAD to NADH, and the oxidative decarboxylation of isocitrate to α-ketoglutarate, respectively.

Mutations in *SDH*, *FH*, and *MDH2* TCA-cycle-related genes lead to the accumulation of its substrates which act as oncometabolites: succinate, fumarate, and malate, respectively. In addition, mutated *IDH1* adquire a neomorphic enzyme activity that converts alpha-ketoglutarate to 2-hydroxyglutarate, another oncometabolite. These metabolites cause hypermethylation by inhibiting 2-oxoglutarate-dependent dioxygenases, such as PHD and histone and DNA demethylases. Thus, on the one hand they act as a competitive inhibitor in the process to hydroxylate HIF prolyl residues, stabilizing HIFα and, mediated by the pVHL, activating genes that facilitate angiogenesis, anaerobic metabolism, and a pseudo-hypoxic state[84,106–108]. On the other hand, due to histone and DNA demethylases inhibition, tumors with mutations in these genes show a similar CpG island methylator phenotype (CIMP) characterized by DNA hypermethylation[32,48,72].

· CLUSTER 1B: von Hippel–Lindau and PGL–polycythemia syndromes

Cluster 1B is characterized, similarly to cluster 1A tumors, by the activation of the pseudo-hypoxia signaling pathway through the stabilization of HIF transcription factor proteins with increased angiogenesis as well as cell proliferation, invasiveness, and migration. However, they do not present the hypermethylation seen in cluster 1A tumors. This cluster contains tumors with mutations in *VHL, EGLN1, EGLN2,* and *EPAS1.* Mosaic (at least in *VHL* and *EPAS1*) and germline mutations in these genes can be associated to the presence of polycythemia.

Mutations in *VHL*, as well as in *EGLN1/EGLN2*, disrupt the process of HIFα degradation, leading to its stabilization, whereas gain of function mutations at *EPAS1* hydroxylation sites disrupt the recognition of EPAS1 by members of the PHD family, as well as its hydroxylation and the consequent degradation by pVHL. To note, PPGL became the first tumors known to carry

activating mutations of *EPAS1*, which had long been implicated in multiple human cancers, but had never been genetically proved to function as a bona fide oncogene[33].

1.5.2 CLUSTER 2: Kinase signaling cluster

As mentioned before, cluster 2 is characterized by the activation of RAS/RAF/MAPK and PI3K/AKT/mTOR signaling pathways and protein translocation, causing a pro-mitogenic and anti-apoptotic state. This cluster contains tumors with mutations at least in *NF1, RET*[88,89], *TMEM127*[100], *MAX*[51], *HRAS*[79], and two genes with a rare involvement: *KIF1B*[59] and *MET*[30].

-. Neurofibromin (NF1) suppresses cell proliferation by promoting the conversion of RAS into its inactive form, thereby inhibiting the oncogenic RAS/RAF/MAPK signaling cascade, and also inhibits the PI3K/AKT/mTOR pathway via suppression of RAS. Thus, *NF1* mutations lead to the activation of both pathways. To note, *NF1* has one of the highest rates of spontaneous mutation of any gene in the human genome[5]. This in part explains why between 30 and 50% of patients have *de novo* mutations[5], and is the gene with the highest rate of somatic mutations[73,96].

-. *RET* encodes a transmembrane tyrosine kinase receptor (RTK) for members of the glial cell line-derived neutropic factor. It activates multiple intracellular pathways involved in cell growth and differentiation. Oncogenic activation of *RET* activates both RAS/RAF/MAPK and PI3K/AKT/mTOR -dependent cell signaling genome[5]. Interestingly, gain of function mutations are related with PCC and medullary thyroid carcinoma (MTC), and inactivating mutations are related to Hirschsprung's disease (HD), but some overlap has been described between MEN2 and HD.

-. *TMEM127* encodes a transmembrane protein which acts as a negative regulator of mTOR. Thus, mutations in *TMEM127* results in reduced inhibition of the mTOR pathway in a RAS/RAF/MAPK and PI3K/AKT independent manner[5].

-. *MAX* encodes a transcription factor, MAX, that belongs to the basic helix–loop–helix leucine zipper family and plays an important role in regulation of cell proliferation, cell differentiation and apoptosis, as a part of the MYC/MAX/MXD1 network. Heterodimerization of MAX with MYC family members results in sequence-specific DNA-binding complexes that act as transcriptional activators. In contrast, heterodimers of MAX with MXD1 family members repress transcription of the same target genes by binding to the same consensus sequence, and thus antagonize MYC–MAX function. Mutated *MAX* causes deregulation of the MYC–MAX–MXD1 pathway that leads to altered transcription and signaling in the NRAS–PIK3CA–AKT1–mTOR pathway. *MAX*-mutated tumors have a unique transcriptomic signature, supported by their intermediate expression of PNMT, and consequently a subsequent lower production of epinephrine[51,84].

-. *HRAS* gene encodes a small GTP-binding protein that affects multiple downstream pathways related to cell growth and homeostasis.

-. *KIF1B* and *MET* are kinesin related genes. While one of the splice variants of *KIF1B,* KIF1Bb, functions as a tumor suppressor that is necessary for neuronal apoptosis, *MET* is a member of the RTK family, but their specific role need further studies.

1.5.3 OTHER GENES

Other genes encoding kinases (*FGFR1)*[93]*,* chromatin remodeling proteins (*ATRX*[102,109]*, H3F3A, KMT2D*, *SETD2*, *JMJD1C*, *KMT2B*, or *EZH2*[93]), and related with multiple type of human neoplasia (promoter region of *TERT*, or somatic mutations in *TP53* and *BRAF*) have been also involved in PPGL pathogenesis*,* but their specific roles have also to be clarified in larger series.

1.6 GENETIC DIAGNOSIS

On the whole, hereditary and somatic mutations explain at least 60-80% of PPGL cases and are found in a mutually exclusive manner[33]. Exceptions to this rule are mutations described in the "new" PPGL-related genes (e.g. *ATRX*), as they have been mainly described in cases with mutations in classical PPGL driver genes, and double somatic mutations described at least in *NF1*[74]and *EPAS1*[98,110]*,* or somatic mutations in *NF1* in tumors carrying a somatic mutation in *RET* or *VHL*[74]. However, these second variants seem to act as modifiers and their role should be resolved by large-scale sequencing analyses[33].

Therefore, current guidelines indicate consideration of genetic testing in all patients with PPGLs, but for cases with indicators of low heritability (unilateral PCC without syndromic features, metastatic presentation, or family history of PPGL), the decision to perform germline genetic testing should be balanced between the cost of testing and the psychological impact on the patient and their family of not having a test that might explain why they have the disease[8].

However, as the genetic spectrum increases with newly described genes having low prevalence (<1% of cases) and no distinctive clinical features, systematic genetic screening of all PPGL-related genes has become a time- and resource-consuming process. The decision of which gene to test is made on the basis of clinical presentation (age at onset, location and number of tumors, syndromic features, family history, and metastases), biochemical secretory phenotype, and immunohistochemical tumor characterization[1,111]. In this regard, many different algorithms have been proposed[35,38,111–116]. In addition, some specific algorithms focused on sporadic PPGL[10,11,36,38–40] have been also proposed, as these cases tend to be excluded from comprehensive genetic screening beyond *SDHB* mutations, and even *SDHB* study is not always performed, being genetic data about sporadic cases still scarce. Importantly, none of the

algorithms proposed contemplate testing for somatic mutations, despite they have been also related to metastatic[73,74,96], and pediatric cases[73], as well as PPGL cases diagnosed before 40 years old[73,78,79,97,117].

1.7 CLINICAL PRESENTATION

In the case of sympathetic tumors (PCCs, TA-PGLs) the clinical presentation is related to the hypersecretion of one or more catecholamines: epinephrine and/or norepinephrine. Later, the enlargement of the tumor can cause mass-effect symptoms in adjacent tissues and organs (e.g. hydroureteronephrosis or renal hypertension)[5]. On the other hand, parasympathetic tumors (HN-PGLs) rarely produce significant amounts of catecholamine (<5%), and commonly present as slow-growing painless cellular masses, being the initial clinical presentations symptoms of cervical mass and/or compression or infiltration of adjacent structures (e.g. hearing loss, tinnitus, cervical mass, dysphagia, cranial nerve palsies)[2,4,5,8].

The classic triad of PPGL symptoms described is headache, sweating, and palpitations, but it only occurs in 40% of the patients. Many patients present arterial hypertension (85-90%), which may be sustained (50-60%) or paroxystic (50%). Peculiarly, hypertensive crises could come up due to incidental tumor manipulation during diagnostic procedures, after using certain drugs, ingestion of foods or beverages containing tyramine, and especially common in children are exercise-induced crises[7]. Other symptoms include pallor (30-60%), feelings of anxiety or panic (20%), fever (66%), or nausea and vomiting (26-43%).

PPGL symptoms and signs are non-specific and can mimic many other conditions, and can vary greatly from one patient to another, even within the same family. PPGL diagnosis is challenging and critical, as un- or miss-diagnosed patients can suffer severe consequences of hypertensive crises, including heart attacks, strokes, and even death[3,66].

1.8 DIAGNOSIS

1.8.1 BIOCHEMICAL STUDIES

Diagnosis of PPGL relies on biochemical evidence of catecholamine tumor secretion. Biochemical testing should be performed in symptomatic patients, patients with an adrenal mass incidentally found during imaging studies or surgery for other reasons, and those who have hereditary predisposition or syndromic features suggesting hereditary PPGL.

Catecholamines are metabolized within chromaffin cells to metanephrines (norepinephrine to normetanephrine, and epinephrine to metanephrine, respectively) and this intra-tumor process occurs continuously and independently of the exocytotic catecholamine release, providing and advantage for measurement of metanephrines during diagnosis of tumors that only release

catecholamines episodically or in low amounts. Measurement of metanephrines in urine and/or plasma has a superior diagnostic sensitivity (97% and 99%, respectively) over measurement of the parent catecholamines. Consequently, metanephrines' measurement remains recommended as the initial screening test (**Figure 3**).

Secretion is so rare in HN-PGLs (<5%), that if a HN-PGL presents hypersecretion it is recommended to discard a concurrent PCC and/or TA-PGL. However, 3-MT, previously mentioned as related with metastatic PPGL, has been shown to be elevated in almost one third of patients with HN-PGLs, and its determination is a useful diagnostic test[4,118].

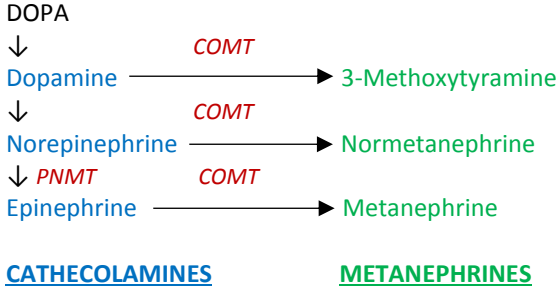


Figure 3. Cathecolamine synthesis and O-methylation.
COMT, catechol-O-methyl-trans-ferase; PNMT, phenyl-ethanolamine-N-methyl-transferase.

Therefore, current recommendations are that initial screening test for PPGL must include measurements of fractionated metanephrines (metanephrine, normetanephrine, and 3-MT) measured separately in plasma, urine, or both, as available, using liquid chromatography with tandem mass spectrometric or electrochemical/fluorometric detection methods, being immunoassays methods a secondary measurement option. To minimize false-positive results, blood sampling should be performed at a supine position (collected after 30 min of supine rest), and overnight fast only when measurements include plasma free 3-MT[118,119]. Despite the plasma test offers sensitivity advantages over the urine test, it is rarely implemented correctly, rendering the urine test preferable for mainstream use[118].

The clonidine suppression test can be useful to distinguish true-from false-positive borderline elevations of plasma normetanephrine, but it has not been validated in any prospective study. In the case of mild elevations, wait-and-retest or proceed directly to imaging studies to localize PPGL could be considered[8].

Test results within reference intervals for plasma free metanephrines exclude almost all cases of PPGL. Exceptions include microscopic recurrences or small tumors (<1 cm) found incidentally or during screening because of a hereditary predisposition to PPGLs or history of the disease, HN-PGLs and rare phenotypically immature A-PGLs that despite having large size are non-secreting tumors (silent A-PGLs). According to this latter one, despite not having defects in the mechanisms of storage or secretion of catecholamines, show absence of the tyrosine

hydroxylase and do not synthesize catecholamines[120]. However, plasma concentrations of chromogranin A (CgA, a biomarker of NETs) are consistently elevated, indicating that CgA can be used as an alternative biochemical parameter in the setting of silent PGLs[120].

Metanephrines measurement provides high accuracy for diagnosis of PPGL, but can also be useful for clinical decision-making about imaging studies during the primary diagnosis and the follow-up. Metanephrine alone, or in combination with normetanephrine, almost always indicate an adrenal location or reflect recurrence of a previous adrenal tumor[121]. Solitary increases of normetanephrine cannot be used to predict tumor location, however the elevation of 3-MT points extraadrenal location[122] and the possibility of metastases[13,14]. Although not offering sufficient power to identify all metastatic patients, plasma 3-MT shows a diagnostic sensitivity of 86% and specificity of 96%, but its measurement is not yet widely available[14,23,36,118].

In addition, as mentioned before, biochemical phenotype can be used to guide genetic testing. For instance, *SDHB* mutation testing has no utility among patients with adrenaline-producing metastatic PPGLs[123], but should be considered in the case of 3-MT secreting tumors[118] or in silent PGLs[120].

1.8.2 IMAGING STUDIES

After confirming a PPGL biochemically, anatomical and functional imaging studies are critical for a) primary tumor localization; b) the detection of multiple primary tumors; and c) the detection of metastases. The knowledge of these three points are important to make the optimal treatment decision between curative surgery and palliative treatment options[124,125]. In the case of HN-PGLs, imaging studies are essential to perform the diagnosis in the majority of the cases.

There is not 'gold-standard' imaging technique for all patients with (suspected) PPGL. A tailor-made approach is clearly warranted to assess disease extension at the time of the discovery of the primary tumor and during the follow-up[23], relying on the decision on many factors: 1) clinical parameters, including age, known hereditary syndrome, renal function (to avoid contrast nephropathy), and the anticipated radiation burden; 2) results of previous imaging (tumor size and location, suspicion of metastases); 3) biochemical findings; 4) preference of the patient; 5) the knowledge of the genetic status; and finally 5) the local availability of scanning systems and insurance issues[125].

1.8.2.1 ANATOMICAL IMAGING STUDIES

First line anatomical imaging modalities include computed tomography (CT) and/or magnetic resonance imaging (MRI), as provide a high sensitivity and allow precise tumor delineation, which is critical for pre-surgical evaluation[125].

CT is the first-choice imaging modality, as it shows an excellent spatial resolution for thorax, abdomen, and pelvis, with a sensitivity between 88 and 100%, being able to detect tumors 5 mm or larger. However, MRI should be considered in the case of HN-PGLs, paracardiac PGLs, and metastatic/residual/recurrent PPGL, as some studies showed that CT-sensitivity was lower than MRI. In addition, MRI is recommended in patients with surgical clips, allergy to CT contrast, and in whom radiation exposure should be limited (children, pregnant/lactating women, and asymptomatic carriers of a germline mutation)[4,8,125]. Despite having high sensitivity, these techniques show a low specificity, making appropriated to complete localization diagnostic procedures with functional imaging studies[2,125]. The combination of anatomical and functional imaging in one time shows the highest sensitivity for the staging of PPGL, but are expensive and not yet widely available techniques.

1.8.2.2 FUNCTIONAL IMAGING STUDIES

The use of functional imaging techniques is recommended in all PPGL, except in the case of PCCs smaller than 5 cm, PPGL associated with adrenergic phenotype and non-*SDHB*[2,25,125]. Different approaches have been described consecutively: planar scintigraphy, single-photon emission computed tomography (SPECT) and positron emission tomography (PET). Each one represents an improvement of the sensitivity and spatial resolution, implying higher price and consequently, a lower availability. To note, PET is also a quantitative imaging technique, as the "Standardized Uptake Value" of the radiotracer can be used to estimate the degree of tracer concentration in a defined region allowing the detection of subcentimetric lesions[125].

The radiotracers used in these techniques are taken up by the tumor cells through different mechanisms that should be known by the physician to decide which type of imaging study should be the more appropriated based on the clinical PPGL scenario.

· **NOREPINEPHRINE TRANSPORTER VIA THE CELL MEMBRANE:** Metaiodobenzylguanidine (MIBG) is structurally similar to norepinephrine. MIBG is commercially available labeled with 123I or 131I. 123I-MIBG in comparison with 131I-MIBG scintigraphy provides images of higher quality, higher sensitivity, and lower radiation exposure. In addition SPECT can be more feasibly performed with 123I-MIBG, and there is less time between injection and imaging (24h versus 48–72h)[25,125]. Thus, 131I is preferable used for targeted radionuclide therapy and 123I for diagnosis and when planning targeted radionuclide 131I-MIBG therapy[25], as besides confirming uptake, it helps achieve personalized dosimetric[25,125]. In the case of PCC, as a diagnostic tool, 123I-MIBG shows a sensitivity (S) and specificity of 85-88% and 70-100%, respectively. However, the sensitivity has been shown to be decreased in PGLs (56-75%), especially in HN-PGLs (18-

50%)[25], and necrotic, metastatic (56-83%), recurrent (<75%), and/or *SDHB*-related PPGLs (<50%)[111,125]. Regarding PET radiotracers, 18F-fluorodopamine (18F-FDA)-PET/CT has the highest sensitivity and specificity across genetically different PGLs (tumors with unknown genotype, *SDHB,* and non-*SDHB)*, and it is the preferred technique for the localization of the primary PGL (S 77–100%) and to rule out metastases (S 77-90%), except in HN-PGLs. 11C-epinephrine[126]and 11C-hydroxyephedrine (11C-HED)[127] are, as FDA, very sensitive and specific radiotracers, but all of them suffer from their limited availability[25,111,125].

· **SOMATOSTATIN RECEPTORS (SSTR):** Overexpression of SSTR-2A and SSTR-3 was recently shown in PPGL with SDH deficiency[128], and different radiolabelled peptides for SSTR have been used not only for the diagnosis, but also when targeted radionuclide therapy with somatostatin analogues (177Lu-DOTATATE) is planned[4,25,125]. 111In-DTPA-Pentetreotide (111In-DTPA-P)/Octreotide (Octreoscan, Covidien) are mainly used in planar scintigraphy, showing lower sensitivity than 123I-MIBG, except in HN-PGLs (S 89-100%)[8,25,125]. 68Ga-labeled somatostatin analogues (68Ga-DOTA-SSTa): 68Ga-DOTA-Tyr3-octreotide (68Ga-DOTA-TOC), -Nal3-octreotide (68Ga-DOTA-NOC), and (Tyr3)-octreotate (68Ga-DOTA-TATE) are used with PET/CT and show sensitivities approaching 100%[129]. They have shown excellent preliminary results in localizing HNPGLs[4], and aggressive and dedifferentiated PPGL[25]. To note, [68Ga]-DOTATATE PET/CT has shown a significantly superior detection rate to all other functional and anatomical imaging modalities in the evaluation of *SDHB* metastatic PPGL[130].

· **GLUCOSE MEMBRANE TRANSPORTER:** [18F]-fluoro-2-deoxy-D-glucose (18F-FDG) accumulates in proportion to the glycolytic cellular rate, providing an index of intracellular glucose metabolism[25,125]. In comparison with other NET that usually exhibit high 18F-FDG uptake in the later stages of the disease, 18F-FDG-PET positivity is almost a constant feature in PPGL (S 74-100%)[125]. It shows a higher performance for metastatic PPGL, and is mainly influenced by the genetic status (e.g. S 83% in *SDHB* versus 62% in non-*SDHB* mutation carriers, being as low as 40% in MEN2-related PCCs)[2,8,22,25,131–133].

· **AMINO ACID TRANSPORTER SYSTEM:** Dihydroxyphenylalanine (DOPA) is the precursor of all endogenous catecholamines, and PPGL cells can take it up through the amino acid transporter system. 18F-FDOPA-PET/CT is an excellent first-line imaging tool, and has a high sensitivity for the localization of non-metastatic PPGL (81-100%), especially in HN-PGLs (100%)[8,25,125,131,134]. In metastatic disease, 18F-FDOPA PET presented higher sensitivity in *SDHB*-negative patients (93%) than in *SDHB*-positive patients (20%)[25,131]. A special advantage in the screening of hereditary

cases is that 18F-FDOPA PET shows lack of significant uptake in normal adrenal glands, very useful for instance in the screening of MEN2 cases[25].

1.8.2.3 OTHER TECHNIQUES

In vivo detection of succinate using pulsed proton magnetic resonance spectroscopy has been reported recently as a highly specific and sensitive hallmark of *SDH*x mutations, being this technique useful to stratify patients or classifying variants of unknown significance (VUS) with no need of tissue sampling. Thus, it may help for the characterization of inoperable tumors and suspicious lesions and serve as a surrogate biomarker in the assessment of tumor response to specific treatments[135,136].

1.8.3 IMMUNOHISTOCHEMICAL TUMOR CHARACTERIZATION

PPGL are positive for CgA, the most reliable marker for discriminating them from adrenal cortical tumors and metastatic tumors that are not NET. PCC may be discriminated from other metastatic NET to the adrenal by staining for tyrosine hydroxylase. Other neural markers such as synaptophysin and neuron specific enolase are typically positive.

Immunohistochemistry (IHC) study could help not only to guide the genetic study, but also to classify VUS identified in the genetic screening. However, they have been only optimized to be used in formalin-fixed paraffin embedded (FFPE) tissue. SDHB-IHC and SDHA-IHC are the most widely used and available techniques, and detect *SDH*x mutations with a high sensitivity and specificity. S*DHB*, *SDHC, SDHD* and *SDHAF2*-mutated tumors are negative at SDHB-IHC and positive at SDHA-IHC, while *SDHA*-mutated tumors are negative at IHC for both[137,138]. Other used IHC have been optimized for identifying truncating *MAX* mutations (MAX-IHC), and S-(2-Succinyl)cysteine (2SC) staining for *FH* mutated tumors. On the other hand, tumors with mutations in TCA genes show almost undetectable nuclear staining of 5-hydroxymethylcytosine (5-hmC), as the accumulation of intermediates associated with their mutations lead to impaired 5-mC hydroxylation[48,55,72].

1.9 TREATMENT

1.9.1 SYMPTOMATIC TREATMENT

An adequate α- and β-adrenergic blockade is needed in PPGL patients at least 2 weeks prior to the surgery, and to control blood pressure and alleviate symptoms related with the catecholamine hypersecretion in those inoperable cases, although they have no effect on tumor size.

Regarding α -adrenergic blockade, phenoxybenzamine is the most commonly used agent, as is a long-acting, nonselective (α1 and α2), and noncompetitive blocker. Doxazosin, prazosin, and

terazosin are specific, cheap, competitive and therefore short-acting α1-adrenergic blockers, but have the potential for severe postural hypotension immediately after the first dose. β-adrenergic blockade using agents such as propranolol, atenolol or metoprolol can be used if the patient present clinical manifestations caused by β-adrenoreceptor stimulations (e.g. tachycardia, arrhythmia, angina, or nervousness). They should be instituted after the α-adrenergic blockade has been optimized (e.g. once the patient develops reflex tachycardia or orthostatic hypotension) as due to the loss of β-adrenoceptor-mediated vasodilatation, an exacerbation of epinephrine-induced vasoconstriction and a resultant serious and life-threatening elevation of blood pressure could occur[3]. Alternative treatments include calcium channel antagonists (e.g. nifedipine and amlodipine), angiotensin receptor blockers, and angiotensin-converting enzyme inhibitors.

On the other hand, α-methyl-para-tyrosine inhibits catecholamine synthesis, but is frequently associated with overwhelming side effects (e.g. anxiety, depression, fatigue, and diarrhea), it is expensive and difficult to obtain. Thus, this medication may be only recommended for selected adults with metastatic PPGL in whom other medications are not able to normalize blood pressure and other symptoms of catecholamine excess[8,23].

1.9.2 SURGERY

The only curative treatment for PPGL is surgery. A minimally invasive procedure using laparoscopic resection is recommended for most PCCs and TA-PGLs if the tumor is small, non-invasive and surgically favorable located. In the remaining cases, open approach should be carried out to ensure complete tumor resection, prevent tumor rupture, and avoid local recurrence. Partial adrenalectomy sparing adrenal cortex could be considered in patients with bilateral PCC or PCC associated with hereditary disease, and those patients with small tumors who have already undergone a contralateral complete adrenalectomy to prevent permanent hypocortisolism[2,111].

Even in cases with advanced disease surgery should be considered, as palliative surgery could release tumor pressure on surrounding tissues or decrease tumor mass (surgical debulking). Despite a survival advantage is not proven, it could also lead to a significant decrease in biochemical secretion, and therefore to decrease α- and β blockade doses to prevent catecholamine release, which can also facilitate subsequent radiotherapy or chemotherapy[2,111].

In the case of HN-PGLs, wait and see may be considered in asymptomatic cases with a low risk of metastases, while active treatment (surgery, radiosurgery or conventionally fractionated

external radiotherapy) is considered in symptomatic cases, in progressive disease, and in cases at higher risk of metastases[4].

Despite there is a vast interest and effort to develop new therapeutic approaches to treat metastatic PPGL, data are either limited or still at an experimental level, as PPGL are tumors characterized by their rarity and heterogeneity[139]. So far, the treatments are basically palliative, and metastatic PPGL is an orphan disease for which therapeutic options are very limited.

1.9.3 INTERNAL TARGETED RADIOTHERAPY

Treatment with 131I-MIBG has been employed to treat metastatic PPGL since 1984 in patients showing positive 123I-MIBG scintigraphy[140]. Although reported therapy effects varied considerably, stable disease could be achieved in 52% and a partial hormonal response in 40%. Reported 5-year survival rate was 45-64% and mean time of progression-free survival 23.1-28.5 months, being hematologic toxicity the most frequent side effect[2,23,141,142]. The use of histone deacetylase inhibitors (e.g. romidepsin and trichostatin A) in vitro and in vivo showed an upregulation of the norepinephrine transporter system, increasing the uptake of 123I-MIBG, that could enhance the therapeutic efficacy of 131I-MIBG treatment[143]. 90Y-DOTATOC, 177Lu-DOTATOC, and 177Lu-DOTATATE treatments have been only used in limited number of patients with positive SSTR-imaging tumors, and more studies should be carried out[23,114,141,144–148].

1.9.4 CHEMOTHERAPY

Combination chemotherapy with cyclophosphamide, vincristine and dacarbazine (CVD) for the treatment of metastatic PPGL was introduced in 1985[149]. CVD is preferred in patients with negative 123I-MIBG scintigraphy and in patients with rapidly growing tumors, even if lesions show positive 123I-MIBG scintigraphy, or extensive organ tumor burden (especially in the liver)[2,23,150]. Partial response could be achieved on tumor volume and hormonal response in 37% and 40%, respectively, but complete response on tumor volume could be achieved in only 4% of patients[2,23,150]. Anecdotally, cyclophosphamide alone achieved a long-term clinical benefit after progression or toxicity with Sunitinib in two frail and symptomatic patients[151].

1.9.5 FOCUSED TREATMENT OF ORGAN METASTATIC LESIONS

External-beam irradiation of bone metastases, especially those that are rapidly growing, or embolization, radiofrequency ablation and cryoablation may provide additional treatment alternatives, not possible if metastases are numerous or very small[2].

1.9.6 MOLECULAR TARGETED THERAPIES

Molecular targeted therapies are promising strategies, but favorable results are still lacking:

-. Everolimus, an inhibitor of mTOR pathway, showed relatively disappointing results in series with few patients[152]. Later, a phase II study reported a modest efficacy, as five of seven patients achieved stable disease[153].

-. Temozolamide and thalidomide, both acting as antiangiogenic agents, in a phase 2 study including only three patients showed an objective biochemical (CgA) and radiological response rate of 40% and 33%, respectively[154]. In a series of 15 cases using temozolamide partial responses were observed in four of 10 patients with *SDHB* mutations and in none of the five patients with sporadic PPGL[155].

-. Imatinib, a selective inhibitor of the ABL, platelet derived growth factor receptor and stem cell ligand RTK exhibited no response in two cases[156].

-. Sunitinib, a RTK inhibitor targeting antiangiogenic factors, has been used in few cases with objective responses and manageable toxicity[157–159]. In a retrospective review of a series of 17 patients, eight experienced benefit according to the "Response Evaluation Criteria In Solid Tumors" (RECIST 1.1) criteria, being the response partial in three and stable in five. In addition, of the 14 patients with hypertension, six became normotensive and two could discontinue antihypertensive treatment. The median overall survival from the time sunitinib was initiated was 26.7 months with a progression-free survival of 4.1 months (95% CI 1.4-11.0). To note, most patients who experienced a clinical benefit were carriers of *SDHB* mutations[160]. Several phase II trials are currently ongoing using RTK inhibitors which endpoints are objective response rate (sunitinib, pazopanib, axitinib, and dovitinib) and progression-free survival (FIRSTMAPP-Sunitinib Trial, www.ClinicalTrials.Gov)[21].

- Somatostatin analogues: octreotide and lanreotide bind with high affinity to SSTR2 and SSTR5 subtypes, and individual reports of octreotide treatment in patients with HN-PGLs have been published[130]. Pasireotide (SOM230), which is active on SSTR 1-3 and 5, showed a more significant inhibition of cell growth, as well as a significantly higher induction of apoptosis in primary PCC cell cultures than octreotide[161]. As SOM230, other treatments have achieved promising results in cellular and animal models, but have no still been used in vivo in humans.

In recognition of the distinct genotype-phenotype presentations of hereditary PPGLs, and the relevance of knowing the gene underlying the PPGL development, a personalized approach to patient management, regarding biochemical testing, imaging, surgery, and follow-up has been recommended[8]. Thus, nowadays it is increasingly evident that successful PPGL management requires a multidisciplinary team approach, and an exquisite genetic characterization of every patient.

II. OBJECTIVES

The main objective of this thesis was to elucidate the genetic heterogeneity of PPGL development in PPGL patients through a systematic genetic screening study. Genetic data were analyzed taking into account clinical parameters, such as number of tumors, age at presentation, and location of the primary tumor, or the presence of metastases among others, to be able to translate this information to useful recommendations for the management of these patients in the clinical setting.

To accomplish this, the study was carried out in two consecutive phases with three objectives each one, respectively:

2.1 PART I:

Genetic characterization of apparently sporadic PPGL (S-PPGL) using Sanger sequencing

- **2.1.1** To assess the prevalence of somatic and germline mutations in the PPGL "major" genes in patients with S-PPGL using Sanger sequencing in DNA samples from blood, FFPE and frozen tumors.
- **2.1.2** To evaluate features classically used to guide the genetic diagnosis in S-PPGL: location of the primary tumor, age at presentation, biochemical secretion phenotype, and presence of metastases.
- **2.1.3** To propose a genetic testing algorithm specifically designed for patients with S-PPGL.

2.2 PART II:

Genetic characterization of PPGL patients using targeted gene panels – Next generation sequencing (TGPs).

- **2.2.1** To perform the genetic screening of "major" and "minor" PPGL genes using two customized TGPs in different types of DNA samples (obtained from blood, saliva, FFPE and frozen tumor) from PPGL index patients.
- **2.2.2** To evaluate genetic results with singular clinical features.
- **2.2.3** To optimize and validate the two TGPs results to be used in the clinical setting.

III. MATERIAL AND METHODS

3.1 PART I: Genetic characterization of apparently S-PPGL using Sanger sequencing

3.1.1 PATIENTS

The inclusion criteria for patients with S-PPGL included the coexistence of four points: (1) the presence of a single PPGL (focal and unilateral); (2) the absence of syndromic features of NF1, MEN1, MEN2, and VHL syndrome in the patient and their relatives; (3) the absence of family history of PPGL, and (4) no known genetic mutation (WT). The diagnosis was based on pathological study and plasma or urine catecholamines and/or metanephrines assessment, as well as imaging tests.

A total of 329 unrelated Spanish index cases with S-PPGLs were recruited between 1997 and 2014 at Spanish public hospitals. All patients provided informed consent for genetic diagnosis.

3.1.2 CLINICAL DATA

A complete clinical questionnaire was requested from each patient, and included the following information: gender, age at diagnosis, clinical presentation (referring to the context in which the first suspicion of PPGL arose, classified as incidentaloma if after an imaging study or from a surgical procedure, and symptomatic if adrenergic or due to local mass symptoms), personal or familial history of signs or tumors of PPGL-related genetic diseases (MTC, primary hyperparathyroidism (PHP), gastro-entero-pancreatic tumors, cutaneous or uterine leiomyomas, renal cancer), findings from physical examination (weight, height, arterial tension, Marfanoid habitus, café-au-lait spots, neurofibromas, freckling), biochemistry studies (hemoglobin, hematocrit, calcium, phosphorus, urine calcium, 25-OH-D vitamin, thyrocalcitonin, CgA, predominant biochemical secretion measured either by liquid chromatography with electrochemical detection or tandem mass spectrometry, depending on the center), results from imaging studies performed (including if optic fundus had been performed, and other signs found in image studies like hemangioblastomas, or visceral cysts), tumor location, number of tumors, and metastatic behavior. The time from the initial diagnosis or resection of the primary tumor used to classify metastases was six months, being ≤ six months for synchronous and > six months for metachronous metastases[24]. Distant metastases were documented by imaging tests and pathological examination when possible[24]. The questionnaire also collected data about surgical and nonsurgical treatments, follow-up visits with the results of the monitoring of biochemical and imaging tests. The family pedigree was also drawn. Spanish version of the clinical questionnaire sent to the corresponding physicians is available in **Supplementary data**.

Among the 329 S-PPGL index patients included: 60.8% were PCCs and 39.2% were PGLs. Among PGLS, 47.3% were HN-PGLs, 10.1% were T-PGLs, 41.9% were A-PGLs, and the location of one

PGL was not specified. The median age at onset was 46 (Interquartile range (IQR): 35–59) years and 58% were women.

3.1.3 SAMPLES

Blood sample to perform germline genetic study was obtained from each patient. Physicians were re-contacted to request tumor sample (frozen and/or FFPE) from patients with negative germline genetic screening. Of 99 tumor samples collected, 75 were FFPE and were studied for SDHB-IHC, and SDHA-IHC on tumors testing negative on SDHB-IHC, as previously described[45,138].

3.1.4 DNA EXTRACTION

DNA was extracted from blood following a standard method and from frozen/FFPE tissue using the DNeasy kit (Qiagen Inc.), following the manufacturer's instructions[162].

3.1.5 MUTATION TESTING: SANGER SEQUENCING

Germline DNA from each patient was tested by Sanger sequencing for mutations in *RET* (exons 10, 11, 13–16), *VHL* (all exons, plus the promoter region), *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *MAX* and *FH* (all exons). Testing for gross deletions in *VHL*, *SDH genes*, *TMEM127*, *MAX* and *FH* was done by MLPA (MRC-Holland) or multiplex PCR, as previously described[51,163,164].

The study of somatic mutations in *RET* (exons 10, 11, and 16), *VHL* (promoter region plus exons 1–3), *EPAS1* (exon 12), *HRAS* (exons 2–3), *MAX*, *SDHB*, *SDHC* and *SDHD* (all exons) was carried out based on biochemical secretion and SDHB-IHC result. Hence, tumors with positive SDHB-IHC were studied for *RET* in adrenergic-secreting tumors, and *VHL* in noradrenergic secreting tumors. As the predominant secretion pattern was not been clearly established for *EPAS1* and *HRAS*, and because *MAX*-mutated cases present both types of secretion, all tumors (except one with negative SDHB-IHC) were studied for somatic mutations in these three genes. *SDHB* was studied if SDHB-IHC showed negative, and *SDHC* and *SDHD* were only tested if SDHB-IHC was negative and SDHA-IHC was positive. Finally, we studied somatic mutations in *NF1* (using the primers previously described[165]) in adrenergic PPGL in which frozen tumor sample was available (five tumors).

NF1 was not tested in FFPE samples, as this gene spans 58 exons, and DNA from FFPE tumor samples suffers from low quality and presence of artifacts, such as C>T base substitutions caused by deamination, and strand-breaks. *NF1* was analyzed in one of the tumors by NGS as part of another study (data not shown). Somatic nature of the mutations was confirmed ruling out their presence in germline DNA. Details summarizing the steps of the study are shown in **Figure 4**.

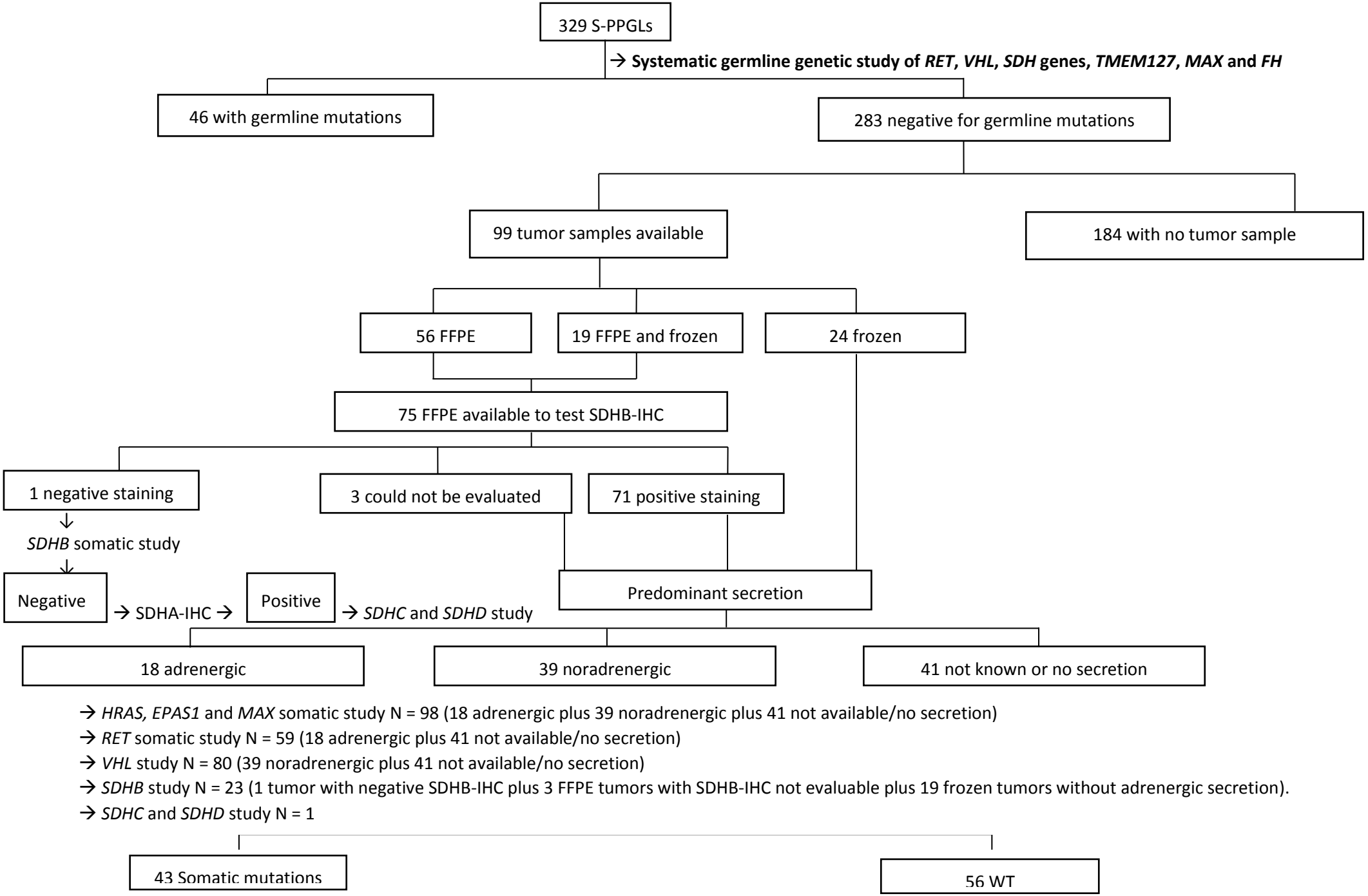


Figure 4. Details summarizing the steps of the genetic workflow study. S-PPGL: sporadic pheochromocytoma/paraganglioma; IHC: immunohistochemistry; FFPE: formalin fixed paraffined embedded.

3.1.6 VARIANT INTERPRETATION

Genetic variants found were classified as mutations or VUS according to information available in public databases. Their presence was checked in the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/); Catalogue of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic), the Single Nucleotide Polymorphism database (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/), Leiden Open source Variation Database (LOVD; http://chromium.lovd.nl/LOVD2/), and the Universal Mutations Database for VHL mutations (UMD-VHL mutations; http://umd.be/VHL/). In silico analysis was performed using Sorting Intolerant From Tolerant (SIFT), Mutation Taster, Polymorphism Phenotyping v2 (Polyphen2), as well as tools able to predict splicing changes. Whether or not the variants had been previously reported was also taken into account.

3.1.7 STATISTICAL ANALYSIS

We used Pearson's $\chi 2$ test, or Fisher's exact test when necessary, to compare proportions. Two-sided p values<0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS statistics V.17.0 (IBM, Armonk, New York, USA) and R software V.2.7.2 (http://www.r-project.org/) was used to generate **Figure 5.**

3.2 PART II: Genetic characterization using TGPs specifically designed for the study of PPGL patients

3.2.1 PATIENTS

The inclusion criteria were patients affected by PPGL. The diagnosis of PPGL, as Part I, was based on pathological study and biochemical secretion of cathecolamines and/or metanephrines, plus imaging tests.

A total of 453 unrelated index patients affected by PPGL were recruited between 1997 and 2016 from 11 PPGL referral centers from Bethesda (USA-Section on Medical Neuroendocrinology, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health) and the European Network for the Study of Adrenal Tumors-ENS@T. Participating ENS@T referral centers were located in Madrid (Hereditary Endocrine Cancer Group, Spanish National Cancer Research Centre), Florence (Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence and Istituto Toscano Tumori), Padova (Endocrinology Unit, Department of Medical and Surgical Sciences University of Padova), Rotterdam (Department of Pathology, Erasmus University Medical Center), Delft (Department of Pathology, Reinier de Graaf Hospital), Liège (Department of Endocrinology, Centre Hospitalier Universitaire de Liège), Dresden (Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, Technische Universitat Dresden), Lübeck (1st Department of Medicine, University Medical Center Schleswig-Holstein, Campus Lübeck), Munich (Department of Internal Medicine IV Campus Innenstadt, University-Hospital, Ludwig-Maximilians-University of Munich), and Würzburg (Department of Internal Medicine I, University Hospital Würzburg).

Amongst included patients, 30 carried pathogenic mutations previously detected by Sanger sequencing (being 13 found in part I), and were used as controls to validate the NGS assay. The remaining cases consisted of 423 unrelated index patients without a known mutation, wild type (WT). In 305 (72%) WT patients, genetic screening by Sanger sequencing had already been partially performed following different algorithms proposed[35,38,111–116] and the genetic workflow study detailed in part I in S-PPGLs (**Figure 4**). The remaining 118 (28%) patients had no previous genetic studies. Clinical characteristics of the 423 WT PPGL patients are summarized in **Table 2**.

All patients provided informed consent for genetic testing. In addition, tumor tissues from the Erasmus MC, Rotterdam, the Netherlands, were used according the code of conduct: “Proper Secondary Use of Human Tissue” established by the Dutch Federation of Medical Scientific Societies.

Table 2. Clinical characteristics of the 423 PPGL patients without a known mutation included in the study.

Type of sample available	Only germline DNA N = 229 (54%)	Germline and tumor DNA N = 27 (6%)	Only tumor DNA. N = 167 (40%)
Classification of the patients based on prior analysis of the samples using Sanger sequencing	Yes, N = 305 (72%): Only germline DNA, N = 215 Germline and tumor DNA, N = 10 Only tumor DNA, N = 80	No, N = 118 (28%): Only germline DNA, N = 14 Tumor DNA available not previously studied, N = 104 Only tumor DNA, N=87 Germline and tumor DNA, N=17	
Patients with syndromic related tumors	N = 13: 4 Medullary thyroid carcinomas or C cell hyperplasia: ID2, D234, ID309 and ID412; 3 Gastrointestinal stromal tumors: ID79, ID95 and ID450; 3 Patients with NF1 clinical diagnosis: ID325, ID332 and ID357; 3 Pituitary adenomas: ID23, ID295 and ID440.		
Family history	N = 5: 1 Patient belonging to a MEN2A family: ID 381; 2 Patients with first degree relatives diagnosed with NF1: ID5 and ID91; 2 Patients with first degree relatives diagnosed with PPGL: ID30 and ID106.		
Sex	Female N = 243 (59%)	Male N = 168 (41%)	
	*No data, N = 12		
Age at onset	Median 48 (IQR = 38-59) years	Pediatric cases (<18 years), N = 13	
Number and location of tumor	Single. N = 362 (88%) PCC, N = 240 HN-PGL, N = 71 TA-PGL, N = 49 Unknown-PGL. N = 2	Multiple. N = 49 (12%) PCC (bilateral and/or multiple), N = 17 PCC and PGL, N = 10 PGL. N = 22	
	*No data N = 12		
Predominant biochemical secretion	- Adrenergic. N = 66 (34%); - Noradrenergic. N = 126 (65%); - Dopaminergic. N = 1 (0.5%): ID401; - Co-secretion of dopamine and noradrenaline/adrenaline. N = 10: ID24, ID107, ID109, ID192, ID284, ID285, ID327, ID402, ID405 and ID 446; Co-secretion of ACTH. N = 2: ID108 and ID304. - Secretion high, but unspecified. N = 21; No secretion. N = 56; Not done. N = 6. *No data. N = 147		
SDHB immunohistochemistry	- Positive. N = 117 - Negative. N = 17	- Not evaluable. N = 2 *No data. N =287	
Metastasis	N = 31 (7.3%)		
Singular pathological features	- Black PCC. N = 2: ID164 and ID429; - Composite tumor with ganglioneuroma. N = 7: ID65, ID100, ID209, ID232, ID294, ID306 and ID435; Composite tumor with lymphoma, N = 1: ID248; - Presence of ACTH in the immunohistochemical study. N = 3: ID108, ID304 and ID451.		

Composite tumor: tumor with presence of neuroendocrine and other type of tumor cells. NF1, Neurofibromatosis type 1; MEN2A, multiple endocrine neoplasia type 2; IQR, interquartile range; PCC, pheochromocytoma; PGL, paraganglioma; HN-PGL, head and neck paraganglioma; TA-PGL, thoracic-abdominal paraganglioma; ACTH, adrenocorticotropic hormone.

3.2.2 CLINICAL DATA

Clinical data in Spanish hospitals were recruited as mentioned in part I with the clinical questionnaire **(Supplementary data)**. Data collected for the other participating centers included at least: number and tumor location, biochemical phenotype, presence of metastases, pathological findings, personal and family history of PPGL or PPGL-related tumors.

3.2.3 SAMPLES

Tumor and germline DNA was requested from each patient. A total of 491 DNA samples from the 453 index patients were studied. DNA obtained exclusively from tumor was available for 182 (40%) cases, matched tumor-germline DNA for 36 (8%) patients, and only germline DNA for 235 (52%) cases. In the latter group, two patients had germline DNAs from two resources: blood and saliva, and blood and GenomiPhi. In only 2 cases germline DNA source was saliva. Of 218 tumor samples, 114 (52%) were frozen and 104 (48%) FFPE. FFPE tumor slides were evaluated for SDHB-IHC, if available.

3.2.4 DNA EXTRACTION

DNA was extracted from peripheral blood samples following a standard method (FlexiGene DNA Kit, Qiagen). For 7 patients, sample material amplified by the Illustra GenomiPhi HY DNA Amplification Kit (GE Healthcare Life Sciences) was used. DNA samples were obtained from saliva using the Oragene·DNA kit (DNA genotek). In tumor samples, the selection of representative tumor areas was performed on a FFPE slide stained with hematoxylin-eosin, if available. DNA from frozen tumor tissue was extracted with the DNeasy Blood & Tissue Kit (Qiagen), and from FFPE tumor tissue with Covaris S2 System (Covaris), according to the instructions provided by the manufacturer. DNA quality was assessed using the NanoDrop spectrophometer, considering an absorbance ratio >1.7 to be acceptable for both 260/280 and 230/260 nm measurements. DNA was quantified with the Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific). The Agilent 2100 Bioanalyzer System (Agilent) was used to assess the size and quantity of DNA fragments in FFPE DNA samples.

3.2.5 MUTATION TESTING: TGPs

3.2.5.1 TGPs DESIGN

Two TGPs were designed using the TruSeq Custom Amplicon 1.5 kit system (Illumina), one (P-I) to work with germline and frozen tumor DNA, and the other (P-II) was a double strand design specifically addressed to study DNA from FFPE tumor tissues, as lead to avoid deamination artefacts. Probes were designed using the online DesignStudio software (Illumina) to capture the coding plus 50 bp intronic flanking regions, excluding non-coding exons. Both designs

contained *RET* (exon 8 to 16), *VHL* (promotor to exon 3), *NF1* (all exons), *MAX* (exon 1, and 3 to 5), *TMEM127* (exon 2 to 4), *SDHA, SDHB*, *SDHD, SDHC, SDHAF2, MDH2, FH* (all exons), *EPAS1* (exon 9 and 12), and *HRAS* (exon 2 and 3). P-I additionally included *KIF1B, MEN1, EGLN1* (all exons), and *EGLN2* (exon 2 to 6). As the involvement of exon 7 in *RET*[166] was not known when TGPs were designed, and it was analyzed by Sanger sequencing.

Table 3. Characteristics of the TGPs designed.

	Panel I	**Panel II**
Type of DNA sample	Germline and frozen	FFPE
DNA input	150 ng	250 ng
Number of genes included	18	14
Type of design	One strand	Double strand
Read Length	2x250 bp	2x150 bp
Amplicon Length	250 bp	150 bp
Number of amplicons designed	344	399 (x2)
Number samples/flow cell	96	48

bp: base pairs; FFPE: formalin fixed paraffined embedded tumor sample.

DNA libraries were prepared according to the manufacturer's protocol and samples were sequenced using the MiSeq platform (Illumina) with a paired-end mode using MiSeq Reagent Kit V3 (Illumina, Spain), 500 cycles in P-I and 300 cycles in P-II. The genetic study of tumor DNA (if available) was prioritized to constitutional DNA.

3.2.5.2 TGPs DATA ANALYSIS

Sequencing reads were de-multiplexed using MiSeq Reporter (Illumina). For raw variant calling, we used Genome Analysis Toolkit v2 (GATK) in P-I and Somatic Variant Caller in P-II. Variant calling format (VCF) was annotated using the version 83 of Ensembl Variant Effect Predictor and assembly GRCh37/hg19 of the human reference genome.

In P-II we doubled checked variants annotated as having a biased prevalence in one of the pools (pool bias), and recovered those previously filtered out due to low coverage in one of the pools if they were detected in at least 20 reads and in 10% of reads. In addition, short indels were detected considering a variation cutoff of 15% in the number of reads in consecutive nucleotides, as problems with these type of variants and MiSeq Reporter had been previously described[98]. We analyzed sequence data using an in-house pipeline. All filtered variants were validated by Sanger sequencing, and the somatic nature was confirmed using constitutional DNA.

In addition, to avoid false negatives results, exons with less than 50-fold coverage were analyzed by Sanger sequencing in samples without mutations found. Additionally, as gross deletions cannot be accurately detected by this platform[94], MLPA and/or multiplex PCR were applied to germline DNA if no mutation was found for *VHL*, *SDH* genes, *FH*, *MAX*, *TMEM127* and *MDH2,* as previously described[51,52,72,94,163,164].

3.2.6 VARIANT INTERPRETATION

The workflow used in the filtering process of sequence data analysis, the study of low coverage regions and gross deletions, and the variant interpretation is depicted in **Figure 6**.

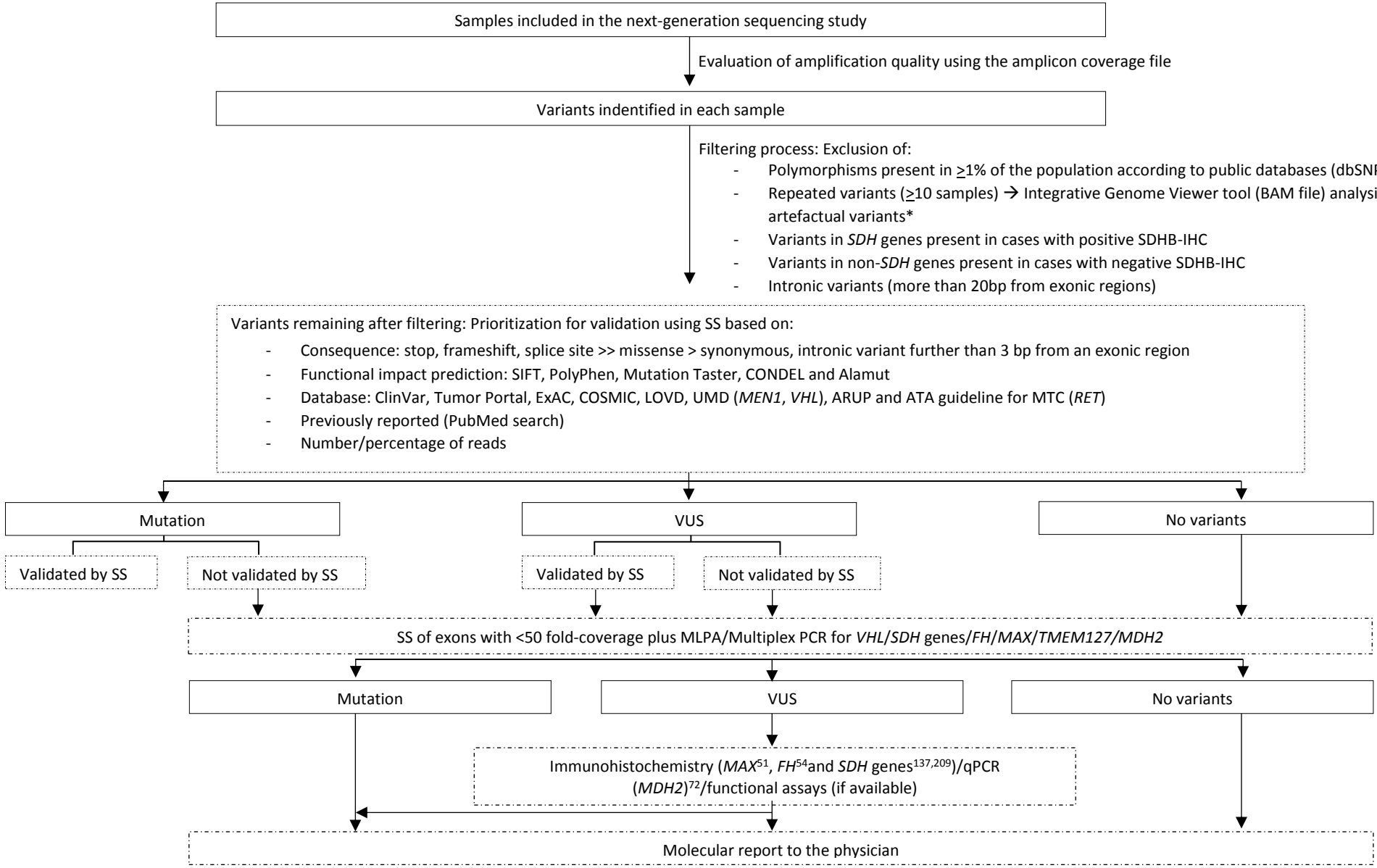


Figure 6. Workflow for next-generation sequencing-based diagnostic testing.

*Artefactual variants are those located in GC rich regions and/or homopolymeric tracts. IHC, immunohistochemistry; EVS, Exome Variant Server; bp, base pairs; CONDEL, CONsensus DELeteriousness score; ExAC, Exome Aggregation Consortium; COSMIC, Catalogue of Somatic Mutations in Cancer; LOVD, Leiden Open (source) Variation Database; UMD, Universal Mutation Database; ARUP, ARUP Scientific Resource for Research and Education (MEN2) RET database; ATA, American Thyroid association- Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma (MTC); UMD, The Universal mutation Database; VUS, Variant Unknown Significance; SS, Sanger sequencing; MLPA, Multiplex Ligation-dependent Probe Amplification assay (MRC-Holland); qPCR, quantitative PCR.

IV. RESULTS

4.1 PART I: Genetic characterization of apparently S-PPGL using Sanger sequencing

4.1.1 CLINICAL CHARACTERIZATION

Clinical characteristics of cases included by tumor location are detailed in **Table 4.** Clinical and genetic data from the 329 patients included in the study are shown **in supplementary table S1.**

Table 4. Clinical characteristics by tumor location.

Location	N	Gender		Age at onset in years	Clinical Presentation		Biochemical secretion		Metastatic cases
		Male	Female	Median (IQR)	Incidentaloma (image/ surgery)	Symptomatic (adrenergic/ local mass)	No	Yes	
PCC	200	88	112	45 (36-57)	41 (36/5)	100 (96/4)	5	124	11
P-value PCC vs PGL				NS			$2.3x10^{-12}$		0.0083
PGL	129	50	79	48 (33-60)	24 (13/11)	62 (26/36)	35	47	18
· HN-PGL	61	21	40	52 (39-61)	5 (2/3)	29 (2/27)	28	7	5
P-value HN-PGL vs T-PGL				NS			NS		0.012
P-value HN-PGL vs A-PGL				0.0057			$3.6x10^{-11}$		NS
· T-PGL	13	3	10	50 (32-62)	4 (2/2)	4 (3/1)	5	5	5
P-value T-PGL vs A-PGL				NS			0.0028		0.046
· A-PGL	54	26	28	44 (24-59)	15 (9/6)	29 (21/8)	2	35	7
· Un-located PGL	1		1	62	unknown		unknown		1
Total cases	329	138	191	46 (35-59)	65 (49/16)	162 (122/40)	40	171	29

NS: no statistically significant differences.

The clinical presentation of S-PPGLs was mainly symptomatic (71.4%). Adrenergic symptoms were the predominant clinical presentation in PCCs (68.1%), T-PGLs (37.5%) and A-PGLs (47.7%), while local mass symptoms were more common in HN-PGLs (79.4%). PCCs and A-PGLs were predominantly secreting tumors (96.1% and 94.6%, respectively), HN-PGLs were more often non-secreting tumors (80%) and T-PGLs were a more even mix of both. Although the proportion of the secreting HN-PGL seems to be higher than previously described[167], it is important to note that we did not have this information for all patients. In these patients with secreting HN-PGL, additional PPGLs were ruled out using images techniques. Fifteen pediatric cases (diagnosed at or under the age of 18 years) were recruited, all derived from sympathetic lineage: seven PCCs, one T-PGL and seven A-PGLs, and most (83.3%) presenting with adrenergic symptoms. Twenty-

nine cases had developed metastases, which were more common in PGLs (14.0%) than in PCCs (5.5%; p=0.008), and in T-PGLs (38.5%) than in HN-PGLs (8.2%) or A-PGLs (13.0%) (p=0.012 and p=0.046, respectively).

4.1.2 GENETIC CHARACTERIZATION

Genetic analysis revealed mutations in 89 (27.1%) of 329 S-PPGLs; 46 were germline (14%) and 43 were somatic (43.4% of the 99 tumors tested). A summary of the assessment of each variant found (mutation vs VUS) is shown in **supplementary table S2.** Germline mutations were more prevalent in PGLs (37/129, 28.7%) than in PCCs (9/200, 4.5%) ($p=6.62\times10^{-10}$). The most frequently germline mutated gene in S-PPGLs was *SDHB* (29/46, 63.0%). This result was expected due to the existence of founder effects affecting this gene in the Spanish population[10]. The number of mutations in other genes was six for *SDHD*, two for *SDHC*, two for *RET* and one for *VHL*. Moreover, as previously published, the contribution of mutations in "new" PPGL-genes was minor: three in *SDHA* (0.9%), one in *SDHAF2* (0.3%), two in *TMEM127* (0.6%), and no *FH* and *MAX* mutations were found[35,53,78,94,96–98,168–170].

Among the 99 tumor samples, 68 were PCCs and 31 PGLs. Among the 75 FFPE samples, all except one showed positive SDHB-IHC. Somatic mutations were more prevalent in PCCs (48.5%) than in PGLs (32.3%; p=0.13). *HRAS* was the gene most often somatically mutated (15.3% of the 98 tumors tested), followed by *VHL* (11/80, 13.8%), *RET* (8/59, 13.6%), *EPAS1* (6/98, 6.1%), *SDHB* (1/23, 4.3%), *NF1,* and *SDHD* (one case each, but only 5 and 1 tumor were studied, respectively). **Figure 7** summarizes results of genetic testing.

4.1.3 RELATION TO TUMOR LOCATION

To make a recommendation about which type of sample (germline versus tumor DNA) should be prioritized for genetic screening of the known PPGL-related genes, and to avoid an overestimation of the frequency of patients with somatic mutations, not only the 99 germline negative cases with tumor material available were included in the analysis, but also the 46 germline-positive patients, together referred as bona fide patients. The remaining 184 germline-negative cases were excluded from this study since tumor material was not available and thus, a somatic mutation could not been discarded. Statistically significant differences were found between PCCs and PGLs regarding the proportion of somatic mutation carriers versus germline mutation carriers ($p=6.67\times10^{-8}$). In this subset of patients, somatic mutations were found in 4.2% of HN-PGLs, 0% of T-PGLs, 24.3% of A-PGLs and 42.9% of PCCs. Among all locations, HN-PGLs and T-PGLs were mainly associated with germline mutations ($p=2.0\times10^{-4}$ and p=0.027, respectively) **(Table 5).**

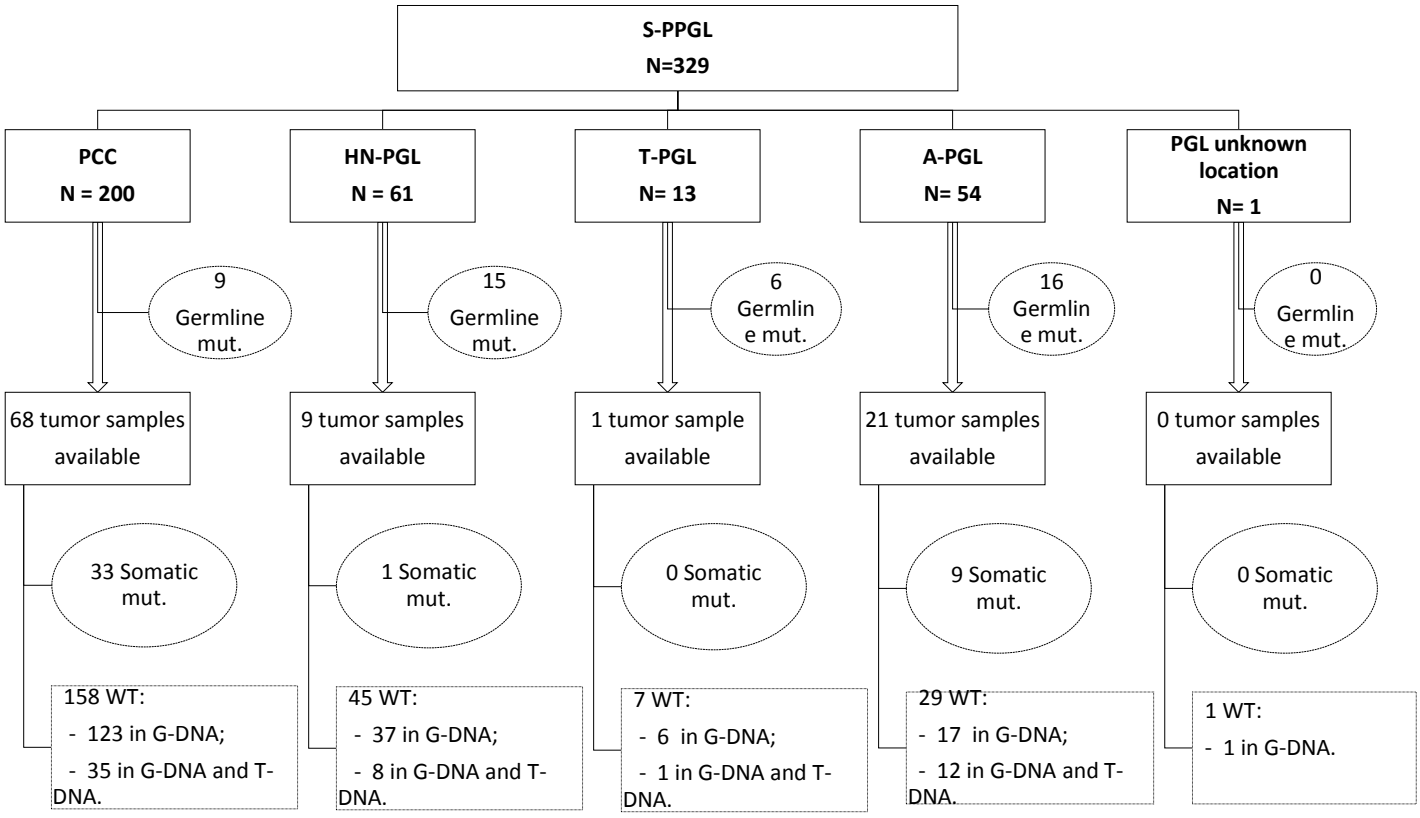


Figure 7. Results for each step of the genetic workflow.
S-PPGL: sporadic PPGL; mut.:mutation; wt: wild type; G-DNA: germline DNA; T-DNA: tumor DNA.

Table 5. Summary of genotype profile by tumor location for cases with germline and tumor DNA available.

Location	Total N = 145	Germline mutation N = 46	P-value Germline vs Somatic mutation	Somatic mutation N = 43	Mutated N = 89	P-value Mutated vs non-mutated	Non-mutated N = 56
PCC	77	9	$6.67x10^{-8}$	33	42	NS	35
PGL	68	37		10	47		21
· HN-PGL	24	15	$2.0x10^{-4}$	1	16	NS	8
· T-PGL	7	6	0.027	0	6	NS	1
· A-PGL	37	16	NS	9	25	NS	12
· TA-PGL	44	22	0.0078	9	31	NS	13

The most frequently mutated gene in PCCs was *HRAS*, while *SDHB* was the major contributor in PGLs regardless of their location, followed by *SDHD* in HN-PGLs, even though the involvement of this gene has been mainly related to multiple PPGLs[33]. In A-PGLs, *SDHA*, *EPAS1* and *HRAS* were

mutated with similar frequency (three cases each). Genetic results by tumor location, and clinical characteristics by gene mutated, are detailed in **Table 6**.

Table 6. Mutations by gene, tumor location and clinical characteristics.

Location	Number of cases (Number of malignant cases)														
	HRAS **N=15 (1)**	***RET*** **N=10 (1)**		***EPAS1*** **N=6 (1)**	***VHL*** **N=12 (1)**		***SDHB*** **N=30 (10)**		***SDHD*** **N=7 (3)**		***SDHC*** **N=2 (1)**	***SDHA*** **N=3 (1)**	***SDHAF2*** **N=1 (0)**	***TMEM127*** **N=2 (0)**	***NF1*** **N=1 (0)**
		Ger. N=2 (0)	**Som. N=8 (1)**		**Ger. N=1 (0)**	**Som. N=11 (1)**	**Ger. N=29 (10)**	**Som. N=1 (0)**	**Ger. N=6 (2)**	**Som. N=1 (1)**					**Som. N=1 (0)**
· PCC	12 (1)	2 (0)	8 (1)	3 (1)	1 (0)	9 (0)	4 (2)	0	0	0	0	0	0	2 (0)	1 (0)
· HN-PGL	0	0	0	0	0	1 (1)	8 (2)	0	5 (1)	0	1 (0)	0	1 (0)	0	0
· TA-PGL	3 (0)	0	0	3 (0)	0	1 (0)	17 (6)	1 (0)	1 (1)	1 (1)	1 (1)	3 (1)	0	0	0
T-PGL	0	0	0	0	0	0	5 (4)	0	0	0	1 (1)	0	0	0	0
A-PGL	3 (0)	0	0	3 (0)	0	1 (0)	12 (2)	1 (0)	1 (1)	1 (1)	0	3 (1)	0	0	0
Gender: Male/Female	5/10	2/0	5/3	0/6	0/1	7/4	18/11	1/0	4/2	0/1	1/1	1/2	0/1	1/1	0/1
Age: Median (IQR)	53 (44-66)	51 (42-61)	48 (43-58)	57 (48-72)	18	27 (17-36)	30 (20-42)	56	34 (28-40)	45	55 (52-57)	26 (21-49)	40	31 (28-33)	51
Secretion: no/yes	3/7	0/5		0/3	0/11		7/14		2/0		1/0	0/3	0/1	0/2	0/1
Type of secretion* N (%)	A 6 (100%)	A 4 (100%)		NAd 3 (100%)	NAd 9 (100%)		NAd 13 (100%)		-		-	NAd 3 (100%)	A 1 (100%)	NAd 1 (50%) A 1 (50%)	A 1 (100%)

PCC: pheochromocytoma; PGL: paraganglioma; HN: head and neck; T: thoracic; A: abdominal; IQR: interquartile range; Ger.: germline; Som.: somatic; A: predominantly adrenergic secretion; NAd. : predominantly noradrenergic secretion.

4.1.4 UTILITY OF PREDOMINANT SECRETION OF PPGLs TO GUIDE GENETIC SCREENING

HRAS-mutated, *RET*-mutated and *NF1*-mutated S-PPGLs presented predominantly adrenergic secretion. *EPAS1*-mutated, *VHL*-mutated, *SDHB*-mutated and *SDHA*-mutated S-PPGLs had, as expected, noradrenergic secretion. Only two *TMEM127*-mutated cases were found, one with adrenergic and the other with noradrenergic secretion. The case in our series with a mutation in *SDHAF2* showed mainly adrenergic secretion.

The result from an assessment of genes mutated by location, for adrenergic and noradrenergic secreting tumors, suggested that in case of noradrenergic secreting tumors, *VHL* should be tested before *SDHB* ($p=3.51\times10-5$) and *SDHD* ($p=7.1\times10-4$) in PCCs, *SDHD* before *VHL* ($p=0.0095$) in HN-PGLs and *SDHB* before *VHL* ($p=0.0024$) in TA-PGLs. However, no statistically

significant differences were found in adrenergic tumors between the proportions of *HRAS*-mutated and *RET*-mutated cases for each location (**Table 7**).

Table 7. Comparison between gene mutated and tumor location.

Location	Number of cases												
	HRAS **N=15**	**p-value *HRAS VS RET***	***RET*** **N=10**	***EPAS1*** **N=6**	**p-value *EPAS1 VS VHL***	**p-value *EPAS1 VS SDHB***	**p-value *EPAS1 VS SDHD***	***VHL*** **N=12**	**p-value *VHL VS SDHB***	**p-value *VHL VS SDHD***	***SDHB*** **N=30**	**p-value *SDHB VS SDHD***	***SDHD*** **N=7**
PCC	12	NS	10	3	NS	NS	NS	10	$3.51x\ 10^{-5}$	$7.14x\ 10^{-4}$	4	NS	0
HN-PGL	0		0	0	NS	NS	0.021	1	NS	0.0095	8	NS	5
TA-PGL	3	NS	0	3	NS	NS	NS	1	0.0024	NS	18	NS	2
T-PGL	0		0	0		NS		0	NS		5	NS	0
A-PGL	3	NS	0	3	NS	NS	NS	1	0.036	NS	13	NS	2

PCC: pheochromocytoma; PGL: paraganglioma; HN: head and neck; TA: thoracic-abdominal; NS: differences statistically not significant (p>0.5).

4.1.5 PEDIATRIC CASES

Data on age at presentation by tumor location and genetic mutational status are summarized in **Figure 5**. The median age at onset for germline mutation carriers was lower than that for somatic mutation carriers and cases without a mutation.

Driver mutations were more frequently found in pediatric than in adult cases (73.3% vs 25.2%, p=0.00020). Germline mutations were found in 53.3% of children, involving *SDHB* in six cases (75%) and *SDHA* and *VHL* in one case each. In addition, three somatic mutations were found in the five tumors available from the pediatric cases with negative germline screening (60%), all of them in *VHL*. Proportionally less adult cases (12.5%) presented germline mutations (p=0.00030), while somatic mutations were found in a similar percentage (41.9%) to that for pediatric patients (p=0.65).

Considering only those cases with bona fide diagnosis (patients with germline mutation and those with negative germline screening and tumor available), 3 (23.1%) pediatric S-PPGL presented a somatic mutation, similar to the 39 (29.8%) somatic mutations found in adult S-PPGL (p=0.15). Similarly, if only pediatric and adult cases with a bona fide diagnosis were taken into account, no statistically significant differences in the proportion of cases with a driver mutation were identified, 11 (84.6%) and 77 (58.7%), respectively (p=0.068).

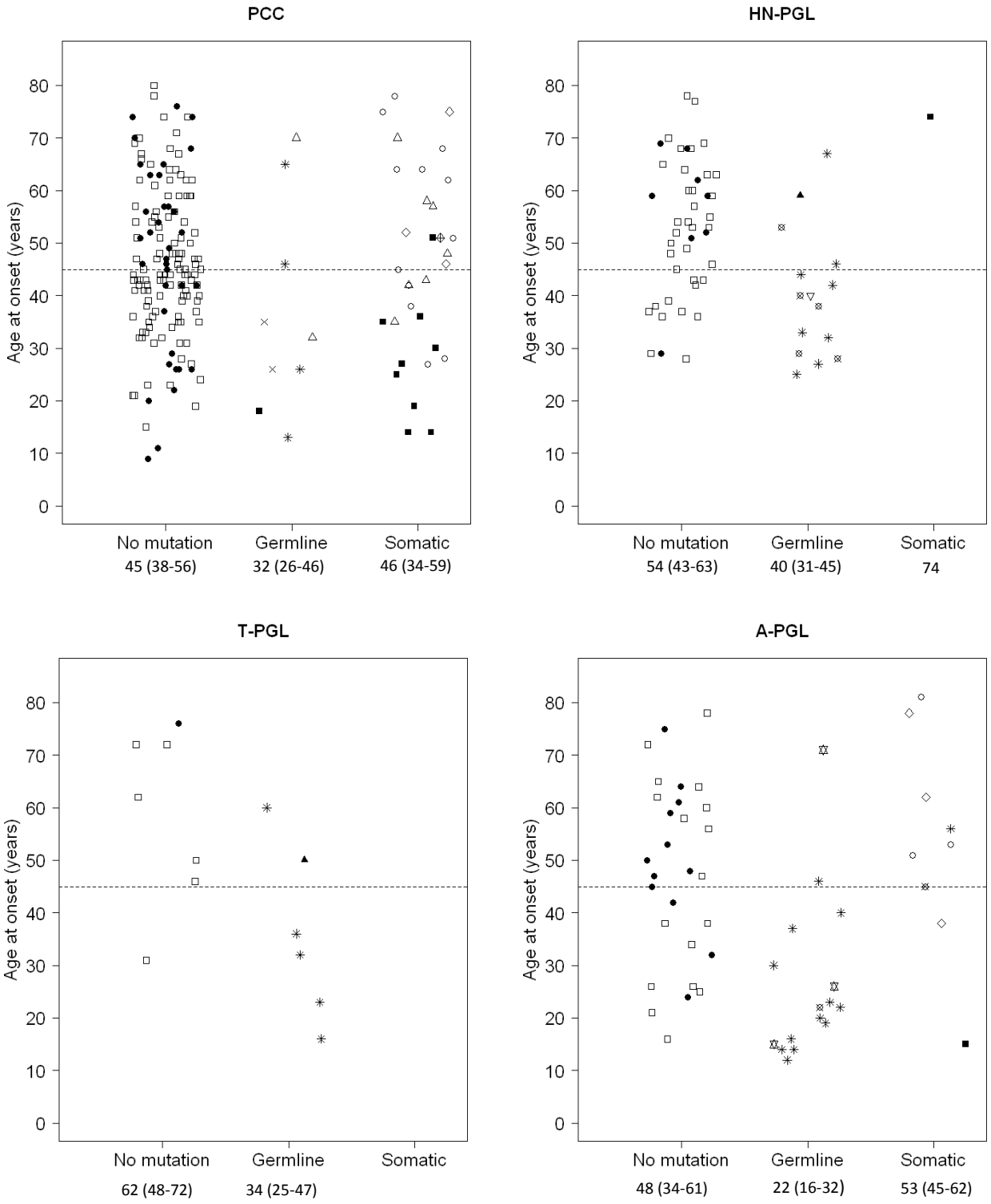
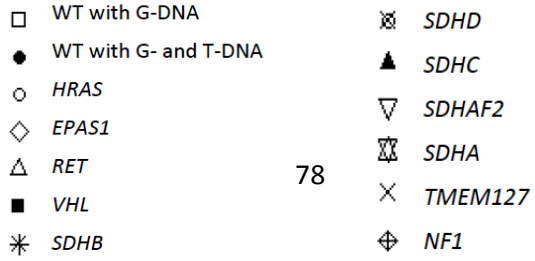


Figure 5. Age at diagnosis by tumor location and genetic mutation status.

WT: wild type; G-DNA: germline DNA; T-DNA: tumor DNA; PCC: pheochromocytoma; HN-PGL: head and neck paraganglioma; T-PGL: thoracic-paraganglioma; A-PGL: Abdominal paraganglioma.



4.1.6 METASTATIC CASES

Of the 29 metastatic cases, 19 (65.5%) harbored a driver mutation. Germline mutations were found in 14 (48.3%), most commonly in *SDHB* (71.4%), followed by *SDHD* (14.3%). No mutations were found in other genes associated with a higher rate of metastases, such as *MAX*[78] and *FH*[53]. However, in one metastatic S-PPGL we found a somatic mutation in *HRAS*, a gene that has not previously been reported to be involved in metastatic PCCs. In addition, as previously described, there were metastatic cases with germline mutations in *SDHA*, *SDHC* or with somatic mutations in *EPAS1*[171], *RET*, *VHL*[73], and *SDHD*[77]**.**

4.2 PART II: Genetic characterization of PPGL using TGPs

4.2.1 TECHNICAL ASSESMENT AND VALIDATION OF TGPs

Good amplification quality was obtained for 466 (95%) DNA samples corresponding to 428 (95%) patients (WT and controls). The NGS assay failed for the remaining 25 samples, despite libraries being generated twice. Since germline DNA was also available for 4 of the tumor samples that failed, they were still included in the study (ID47, ID71, ID101 and ID123). **Supplementary table S3** details clinical characteristics of the 21 remaining patients.

The sensitivity of NGS P-I and P-II was assessed based on polymorphic and pathogenic variants previously found by Sanger sequencing: 534 (73 unique) and 337 (56 unique) for each panel respectively, and reached 99.6% (P-I) and 99.4% (P-II). The only 4 variants not detected by TGPs were located in amplicons showing low coverage (≤ 50 reads): 1 VUS in *TMEM127* (exon 2) and 1 Single nucleotide polymorphism (SNP) in *MDH2* (exon 1) in P-I, and 2 SNPs in exon 1 of *MDH2* in P-II. The assay was still informative in low coverage regions, as 17 SNPs located there were validated **(Supplementary tables S4 and S5).**

Considering both panels, 7% of exons included in the design (16/224 of P-I and 11/157 in P-II) showed low coverage, 38% affecting exon 1 of different genes, and the remaining were located in regions with high GC content, as previously reported[98,172]. Sanger sequencing of low-coverage regions did not detect any variant.

In addition, cross-amplification of *SDHA* and *NF1* pseudogenes was ruled out in both panels since 29 *SDHA* and 3 *NF1* previously known variants were validated by P-I, and 25 *SDHA* variants by P-II. Similarly, 19 *NF1* variants were found using P-II and validated by Sanger sequencing.

The longest duplication detected was 6bp in length (*SDHB:* c.424-19_424-14dupTTCTTC) in both panels. The largest deletions identified by P-I and P-II spanned 6bp (*SDHB*: c.424-19_424-14delTTCTTC) and 22 bp (*NF1*: c.2364_2385delAAAGCTAATCCTTAACTATCCA) in length, respectively. *SDHB* gross deletions were not detected by the NGS assay in a positive control and a new positive case (ID 152).

4.2.2 GENETIC CHARACTERIZATION

4.2.2.1 DETECTION OF VARIANTS IN WT PATIENTS

NGS analysis of the properly amplified 403 WT patients revealed 89 pathogenic mutations (71 unique), 29 germline mutations, 58 somatic mutations, and 2 mutations in tumor DNA of patients without germline DNA available**. Figure 8 and figure 9** detail mutated cases.

The most frequently germline mutated genes were *SDHB* (2.2%, 9/403) and *SDHD* (1.2%, 5/403), followed by *SDHC, FH, NF1* (0.7%; 3 mutations in each gene), *SDHA* (0.5%, 2/403), and finally *SDHAF2*, *VHL, RET* and *MAX* (0.25%; 1 mutation in each gene).

Among the 183 tumor samples of WT patients with properly amplified, *NF1* was the gene most frequently mutated (14%). Somatic mutations in *VHL*, *HRAS* and *RET* were found in a similar percentage (6.6%, 5.5% and 4.4% respectively), and EPAS1 was involved in 3 (1.6%) cases. Of note, one germline DNA and 5 tumors apparently negative by Sanger sequencing showed mutations with low percentage of reads (<15%) by NGS. A review of the chromatograms and/or second tumor selection confirmed the NGS findings by Sanger sequencing (**Figure 10**).

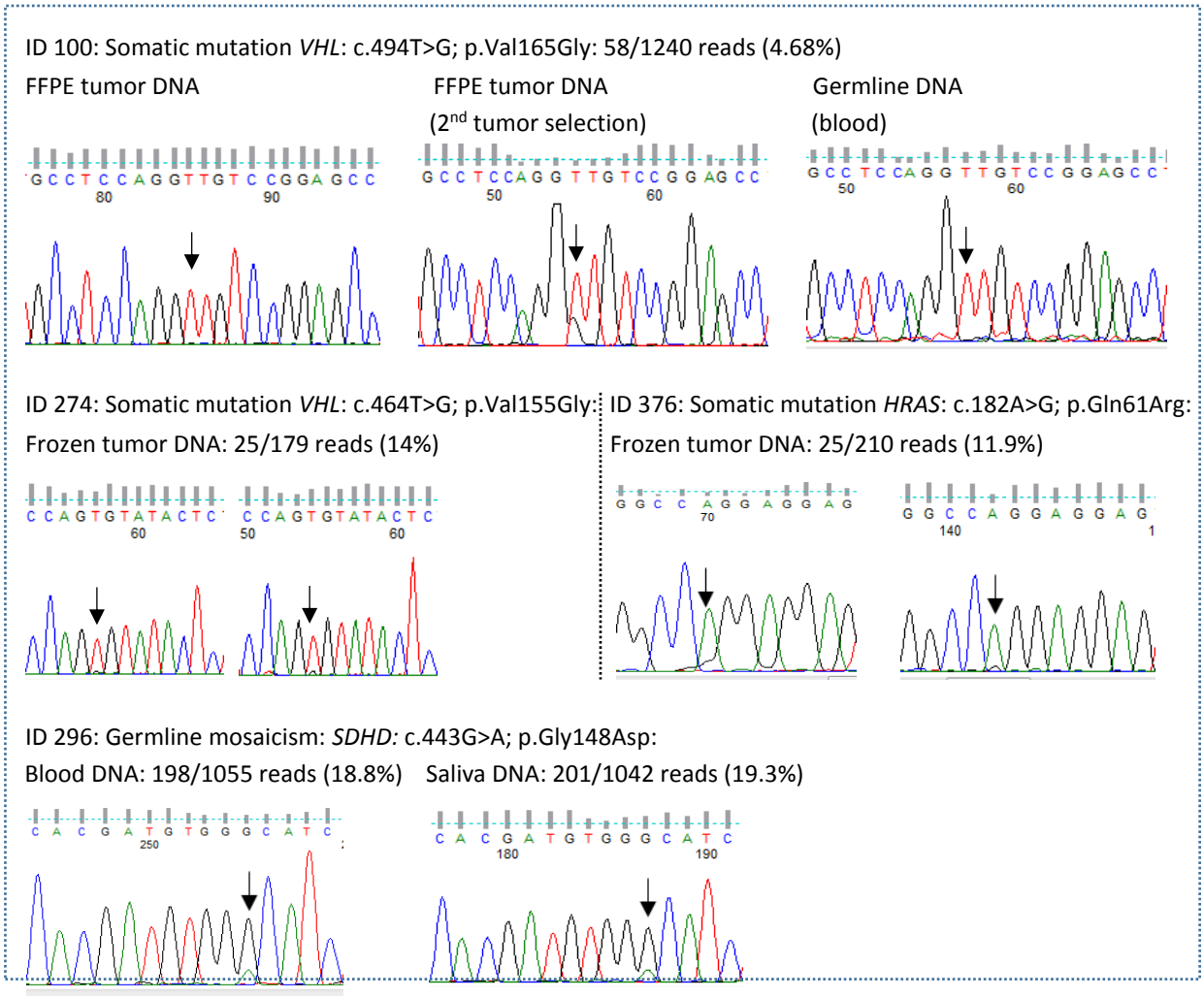


Figure 10. Sanger sequencing chromatograms of pathogenic variants found in low percentage of reads.

Germline mutations were more prevalent in cluster 1 genes (83%), while somatic mutations predominantly affected cluster 2 genes (74%).

In addition, 45 VUS (42 unique) were found, 35 germline and 10 in tumor DNA (2 of them somatic

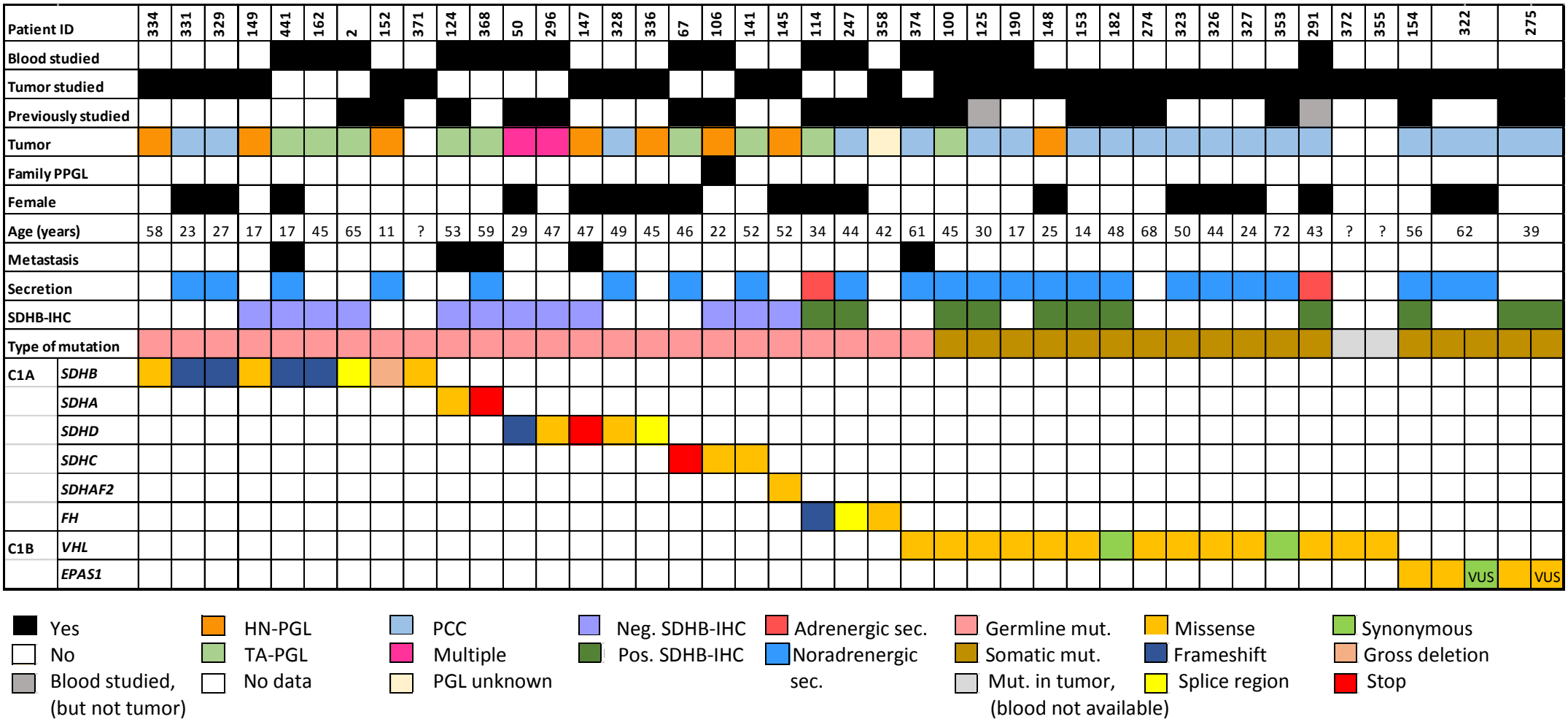


Figure 8. Cluster 1 mutations.

PPGL, pheochromocytoma and/or paraganglioma; IHC, immunohistochemistry; VUS, variant of unknown significance; HN-PGL, Head and neck paraganglioma; TA-PGL, thoracic-abdominal paraganglioma; PCC, pheochromocytoma; Neg., Negative; Pos., Positive; Adrenergic sec., predominantly adrenergic predominant secretion; Noradrenergic sec., predominantly noradrenergic secretion; mut., mutation; Cases categorized as probably germline or somatic, are represented as germline and somatic mutations, respectively.

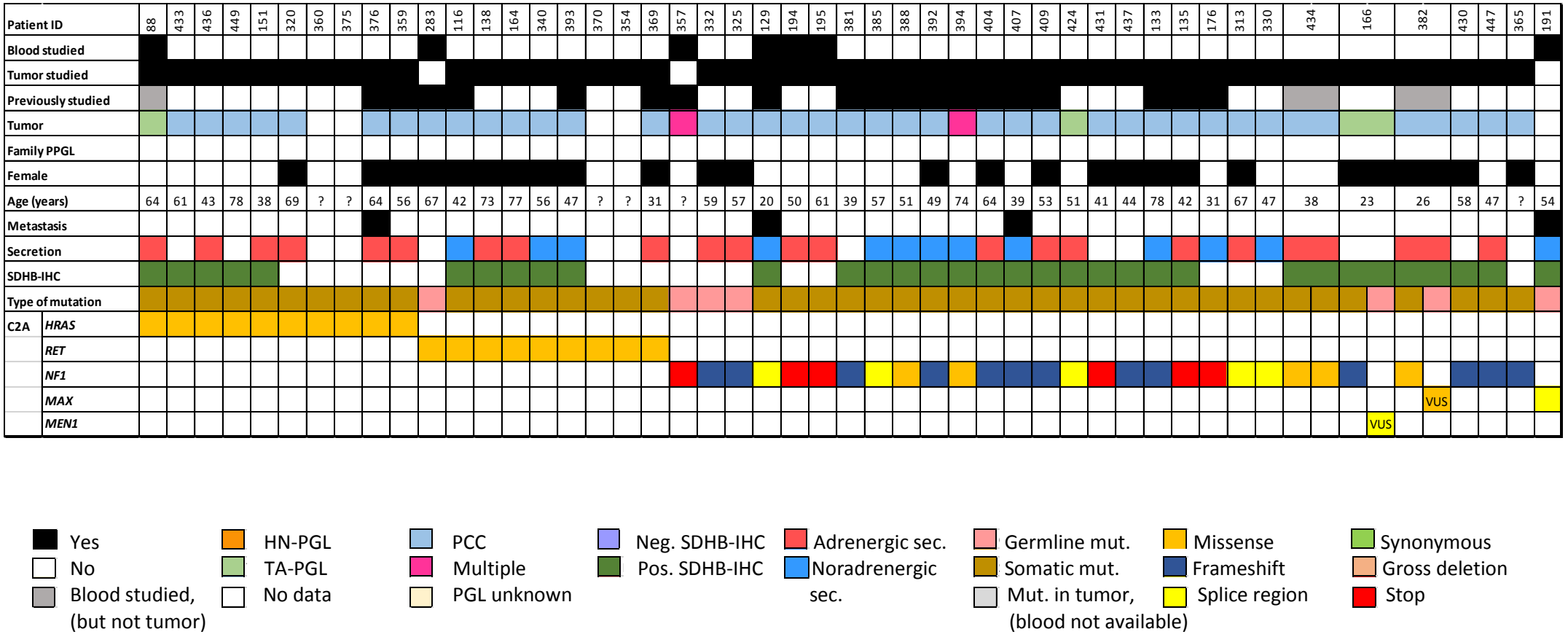


Figure 9. Cluster 2 mutations.

PPGL, pheochromocytoma and/or paraganglioma; IHC, immunohistochemistry; VUS, variant of unknown significance; HN-PGL, head and neck paraganglioma; TA-PGL, thoracic-abdominal paraganglioma; PCC, pheochromocytoma; Neg., negative; Pos., positive; Adrenergic sec., predominantly adrenergic secretion; Noradrenergic sec., predominantly noradrenergic secretion; Mut., mutation; *This VUS in *MAX* had been previously reported and functional assays found that it was not pathogenic.

mutations). Three VUS were found in patients carrying pathogenic mutations. Twelve VUS involved *SDH* genes, but SDHB-IHC could only be performed in two, strongly arguing against pathogenicity, as SDHB-immunostaining was positive. Other VUS involved *NF1* (7), *FH* (5), *MEN1* (2) and *RET* (1); but none of these patients presented with syndromic features. VUS were also found in *EPAS1* (4), *MDH2* (6), *KIF1B* (3) and *TMEM127* (2). A summary of mutations and VUS is shown in **Supplementary table S6.**

To note, among VUS validated by Sanger, five might be pathogenic: a *SDHB* missense variant predicted by in-silico tools to be deleterious and possibly damaging, not previously described and, in which we did not have available FFPE tumor sample to perform SDHB-IHC; a *FH* missense variant associated with positive SDHB-IHC and negative 5-hmC IHC, in which we requested more FFPE slides to perform 2SC-IHC study; a RET synonymous variant described to affect splicing[173]; two candidate second hit *EPAS1* variants, located close to the hydroxylation site in patients carrying known pathogenic *EPAS1* mutations. Thus, further functional assays are required to determine their pathogenicity.

Twenty-four variants reported by NGS were not validated by Sanger sequencing; two were located in homopolymeric tracts in *KIF1B*, and 22 showed low coverage of the variant (<12% and <13 reads of the altered variant), suggesting they were artefacts **(Supplementary table S7)**. For 272 patients no variants were found; tumor DNA was available for 90 (33%) of these.

4.2.2.2 DETECTION OF VARIANTS ACCORDING TO PREVIOUS SANGER SEQUENCING

The sample set (properly amplified) was divided in two groups: 1) cases previously partially studied by Sanger sequencing according to genetic testing algorithms (WT^{ps}; N=289); and 2) patients not previously studied (WT^{notps}; N=114). The distribution of the variants in each group is represented in **figure 11** (mutations) and **figure 12** (VUS).

As expected, driver mutations were more frequently found in WT^{notps} (52/114, 46%) than in WT^{ps} (37/289, 13%). While WT^{notps} had more germline mutations (14%, 16/114) than WT^{ps} (4.5%, 13/289), the percentage of somatic mutations was similar: 34 (34%) of the 100 tumors available in WT^{notps} and 24 (29%) of the 83 WT^{ps} tumors.

Three *FH* mutations (3/289, 1%), two mutations in each of *SDHB*, *SDHD* and *SDHC* (2/289, 0.7%), and one mutation in each of *SDHA*, *RET* and *VHL* (1/289, 0.35%) were found among the WT^{ps}. Among WT^{notps} *SDH* genes were the major players.

All *NF1* germline mutations were found in NF1 syndromic patients, and somatic *NF1* mutations were found in a similar percentage in WT^{ps} (15 %) and WT^{notps} (13%), as *NF1* was not previously studied by Sanger sequencing.

4.2.3 VARIANTS FOUND IN CASES WITH SINGULAR FEATURES

Among the 4 non-*RET* cases with MTC or C cell hyperplasia, only one had a *SDHB* germline mutation (ID2). No mutations were found in patients with GIST nor pituitary adenomas.

A *SDHC*-germline mutation was identified in one (ID30) of the 2 PPGL familial cases. In the 2 patients with NF1-affected relatives, no *NF1* germline mutations were found, suggesting they might be phenocopies. We could not assess this hypothesis, because tumor DNA was not available. Patient ID381, from a MEN2A family, appeared to be a phenocopy due to a *NF1* somatic mutation.

One somatic mutation in *VHL* (ID327) was found among the eleven dopamine-secreting cases, and in one out of the 8 composite tumors (ID100). The two black PCCs harbored *RET* mutations, one (ID164) a *RET* p.Met918Thr somatic mutation and the other (ID429) a germline VUS. No mutations were found in the 3 ACTH-immunostaining positive cases.

4.2.4 MULTIPLE CASES

In the 47 cases with multiple tumors and properly amplified, mutations were identified in 13% (6/47): 1 *NF1* germline mutation in a clinically diagnosed *NF1* case, 3 *SDHD* and 1 *SDHAF2* germline mutations in patients with multiple HN-PGLs, and 1 *NF1* somatic mutation in a reported "double" PCC (out of 15 available tumors).

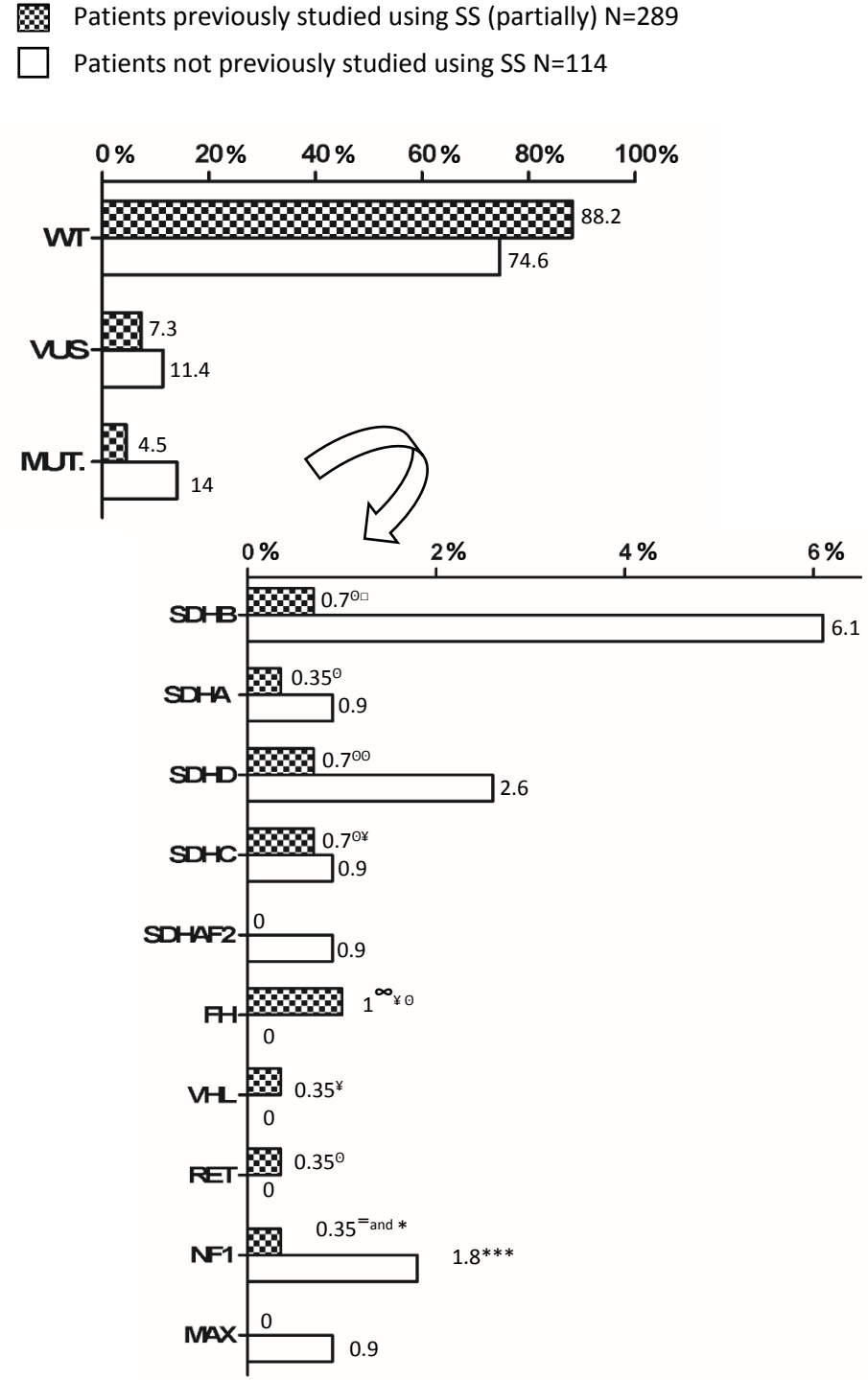
7.2.5 PEDIATRIC CASES

Regarding pediatric cases, a driver mutation was found in 41.7% (5/12): 3 *SDHB* germline mutations and 2 *VHL* somatic mutations (out of the 7 with tumors available, 28.6%). Only one case did not show properly amplified.

7.2.6 METASTATIC CASES

Finally, a driver mutation was detected in 30% (9/30) of metastatic cases well-amplified. Six harbored germline mutations in *SDHA* (2), *SDHB*, *SDHD*, *MAX* or *VHL* (1). Three out of the 16 available tumors (18.8%) harbored somatic mutation in *NF1* (2) and *HRAS* (1). No mutations were found in *MDH2* or *FH*.

GERMLINE MUTATIONS



SOMATIC MUTATIONS

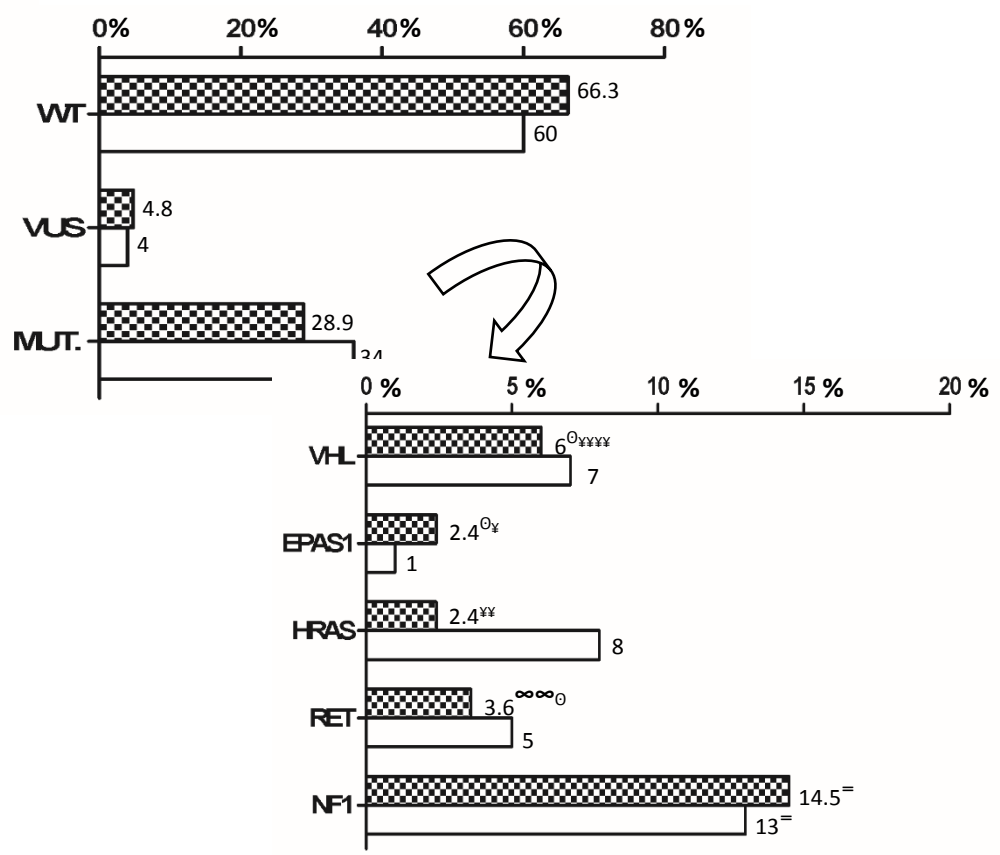
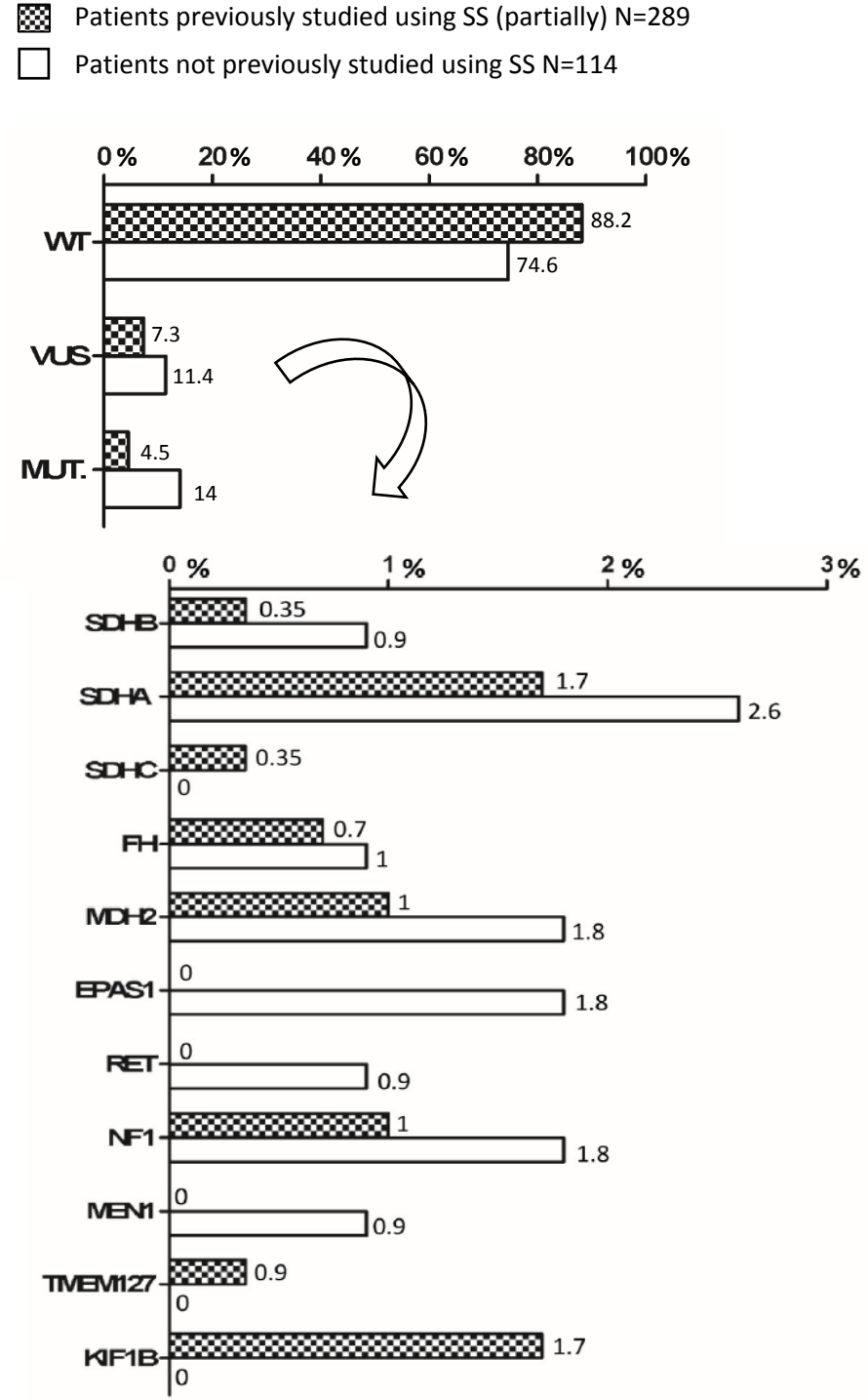


Figure 11. Comparison of the distribution of mutations depending on if the samples had been previously studied (partially) using Sanger sequencing or not.

SS: Sanger sequencing; WT: Wild Type; VUS: Variant of Unknown Significance; MUT. Mutation. Reason of having not considered the study of the gene found mutated in the cases previously studied using SS: Θ: No predominant biochemical secretion data available; ∞: Opposite biochemical secretion data; □: No blood available previously to perform gross deletions, only frozen tumor; ¥: Data from secretion received between SS-MiSeq.; ×: No data received*: Syndromic features; =: Not previously studied using SS.

GERMLINE VUS



SOMATIC VUS

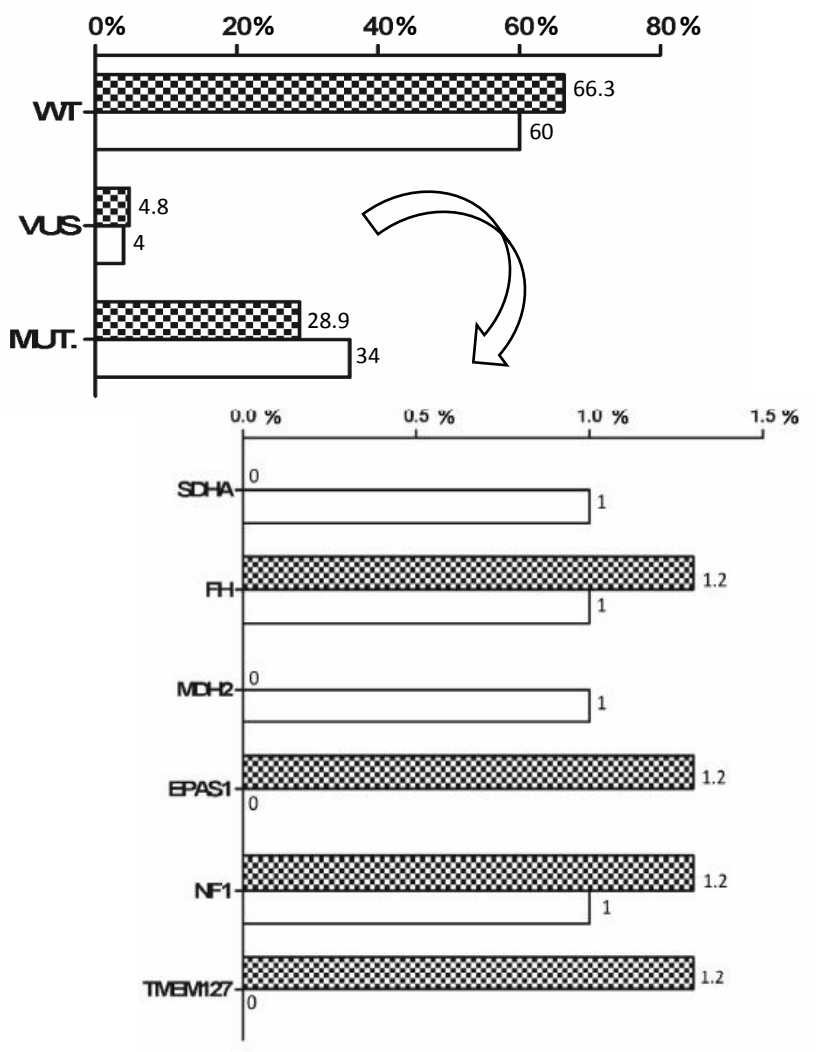


Figure 12. Comparison of the distribution of VUS depending on if the samples had been previously studied (partially) using Sanger sequencing or not.

SS: Sanger sequencing; WT: Wild Type; VUS: Variant of Unknown Significance; MUT. Mutation.

V. DISCUSSION

5.1 DRIVER GERMLINE AND SOMATIC MUTATIONS

5.1.1 GERMLINE MUTATIONS

Since 2002, several reports have been published on genetic screening in S-PPGLs, with the estimated prevalence of hereditary cases ranging from 11.3% to 24%[36–40,115,174]. However, it is difficult to compare these findings because the criteria used to define S-PPGLs varied between studies; some included cases with multifocal[40] or bilateral[36,40] tumors, or cases with family history[40], while others focused on benign tumors[39] or secreting tumors[36,40], or included cases diagnosed within a specific age range[174]. Further, only germline mutations involving the PPGL-related genes known at the time (*RET, VHL, SDHB, SDHD* and *SDHC*) were considered.

That said, in our 2009 study, we found germline mutations in 19 (14%) of the 135 cases studied[38], and part I of this thesis shows the same percentage of hereditary cases (N=46, 14%), despite having increased the sample size to 329 patients and five additional genes having been included in the analysis (*SDHA, SDHAF2, TMEM127, MAX,* and *FH)*, but these results were expected, as these "new" genes have a limited contribution in PPGL susceptibility. In fact, only were involved in three cases for *SDHA* (0.9%), one in *SDHAF2* (0.3%), two in *TMEM127* (0.6%), and no *MAX* or *FH* mutations were found.

TGPs detected germline mutations in 29 (7.2%) PPGL cases, despite we included six more PPGL-related genes in the germline genetic screening (*NF1, MEN1, KIF1B, EGLN1, EGLN2,* and *MDH2)*. This proportion was the expected, since 95% of the patients included in part II were not syndromic and had no family history, and similarly to part I, germline mutations involving the "minor" genes was similar to that reported (<1%)[33,35]: three in *FH* (*0.7%)*, two in *SDHA* (0.5%), and one in *MAX* and *SDHAF2* (0.25%), with no mutations found in *TMEM127, EGLN1, EGLN2, KIF1B, MDH2*, and *MEN1*. The 3 germline mutations in *NF1* were found in patients with previously known clinical features of NF1 syndrome (ID325, ID332 and ID357), as anticipated.

Moreover, if we consider the patients included in part II that accomplish with the criteria used in part I for S-PPGL (single tumors without syndromic features and absence of a family history of PPGL), we analyzed 335 S-PPGL using TGPs in part II. Among them, 18 germline mutations were found (5.4 %): 8 in WT^{ps} (3%) and 10 in WT^{notps} (11.1%), being the rate in WT^{notps} similar to that previously reported in non-syndromic cases[175] and part I.

These results confirm that all S-PPGL should be included in the study of germline mutations, as S-PPGL showed in both parts of this study a rate of germline mutation higher than 10%, the rate stablished by the American Society of Clinical Oncology (ASCO) to consider genetic screening.

In addition, our results confirm the limited contribution reported for "novel" PPGL-genes: *SDHA* (<1%)[33], *SDHAF2* (<1%)[170], *TMEM127* (*0.9%*)[169], *MAX* (*1.1%*)[78] *and FH* (0.83%)[53,94,97,98]. Furthermore, cases related to the remaining relatively "new" genes can be considered anecdotic, as very few patients have been associated so far with germline mutations in them: *EGLN1* (N=2), *EGLN2* (N=1)[58], *KIF1B* (N=2)[59], and *MDH2* (N=1)[72], and we did not discover any other case. To note, *MEN1* mutations have not been identified in S-PPGL[98].

However, despite mutations in these 10 "minor" genes are rare, genetic screening of them (comprising 89 exons) plus *NF1* (comprising 58 exons) by conventional methods would have delayed the diagnosis, which is especially critical for *NF1*[74,176], *MAX*[78], *FH*[54], *SDHA, SDHC*[177,178], and *MDH2*[72] mutation carriers, as mutations in these genes have been associated with metastases and poor prognosis. Thus, these genes should not be excluded from a comprehensive genetic screening in PPGL cases.

5.1.2 SOMATIC MUTATIONS

In recent years, somatic mutations in S-PPGLs have also been reported, highlighting the importance of working with tumor samples to provide a genetic diagnosis[73,74,78–80,95,96,98,99,117].

In part I of this study, somatic mutations were found in 43.4% of the 99 tumors tested, showing a higher frequency of somatic mutations than the previously reported rate 36%[74], probably because we included the study of *HRAS* and *EPAS1*, which seems to be relevant to S-PPGL. A higher frequency of mutations was seen for *RET* (13.6%), *VHL* (13.8%) and *HRAS* (15.3%); as the previously reported prevalences were 5–5.1%, 8.5–9.2% and 6.9–10%, respectively[73,74,79,95,117]. On the other hand, our study found a similar frequency of *EPAS1* mutations (6.1%) to that previously reported 7.9%[80].

Remarkably, although germline mutations in the *HRAS* and *EPAS1* genes have been reported to be associated with the 'Costello syndrome' and 'familial erythrocytosis type 4', respectively, no case with PPGL has been reported in families with those syndromes[33,79,117,168]. However, elevated urine catecholamine metabolites have been described in some patients with Costello syndrome[179] and *EPAS1* mutations have been found as a mosaic in germline DNA extracted from leucocytes and buccal cells in two patients with polycythemia and PPGLs[180,181]. Although we did not ruled out the presence of these mutations in germline DNA in part I, none of the cases with somatic mutations in *EPAS1* or *HRAS* showed any of the associated syndromic features.

Despite somatic mutations in *SDH* genes have been reported very rarely[77,182–186], we found a somatic mutation in *SDHB* and another one in *SDHD* in 2 S-PPGL.

In 2012, two independent studies found somatic *NF1* mutations in 24% and 41% of PPGL patients, predominantly in PCCs and one A-PGL[74,96]. For three of the cases, the mutation was found to be in the germline, all had mild features and none had previously been identified as a syndromic NF1 patient[74]. We found one somatic mutation in *NF1* among the five adrenergic frozen tumors available (20%). This lower percentage is probably due to the limited number of tumors analyzed for somatic *NF1* mutations, but studying *NF1* using Sanger sequencing is difficult due to large size, the absence of identified hot spots, and the high cost and time of delivery.

When using TGPs, somatic mutations were detected in 32% of the 183 tumors studied. If we consider the tumors without germline mutations in part II (N=167), as done in part I, we detected somatic mutations in 35%: 31% of WT^{ps} (25/82) and 39% of WT^{notps} (33/85).

To note, despite being *NF1* the gene most frequently somatically mutated[74,96], the use of TGPs revealed a relatively lower prevalence of somatic mutations in *NF1* (14%), as well as in the other genes somatically involved: *VHL* (6.6%), *HRAS* (5.5%), *RET* (4.4 %), and *EPAS1* (1.6%), in comparison with previously published data: 24-41% in *NF1*[74,96], 8.5–9.2% in *VHL*[73,74,95], 6.9–10% in *HRAS*[79,117], 5–5.1% in *RET*[73,74,95], and 7.9% in *EPAS1*[80]. Although tumors from WT^{notps} showed a more similar percentages to previously reported (**Figure 11**), the lower percentages in general are probably caused by the fact that in previous studies, part I, and WT^{ps} of part II, somatic study was carried out in selected cases using different parameters (e.g. biochemical secretion)[73,79,117,176].

Finally, *MAX* was not somatically involved in our series, which is consistent with previous reports (1.65–2.5% frequency with only three cases reported previously)[78].

5.2 VARIANTS OF UNKNOWN SIGNIFICANCE

One of the main problems of NGS is the amount of data derived of their use, being the finding of numerous VUS a challenge for clinical diagnosis. In comparison with part I, in which only 6 different VUS were found (only one in a case with a pathogenic mutation (ID619)), TGPs elucidated 45 VUS (39 different VUS, being only three present in cases with pathogenic mutations (ID322, ID275, ID166)).

Among the 5 patients in which the only finding in part I was a VUS, four were included in part II (ID130, ID218, ID382, and ID395). ID382-patient presented a VUS in *MAX* in part I (p.Ser142Leu), that afterward was proven to be not pathogenic by our group through functional studies[187], and the inclusion in part II revealed a *NF1* pathogenic somatic mutation. No other mutations were found in the remaining cases, although only one tumor sample could be obtained. The fifth

patient of part I that was not included in part II, presented a missense *SDHB* variant in a highly conserved residue (ID163: p.Asp74Gly) and we requested a slide of FFPE tumor sample to perform SDHB-IHC to assess the pathogenicity of the variant to the corresponding physician. Other IHC previously mentioned can be further used to assess the pathogenicity of the VUS found, such as SDHA-IHC for *SDHA* VUS[138], 5-hmc and 2SC for *FH* VUS[54], MAX-IHC for novel *MAX* truncating variants[51,78] among others.

Thus, to further characterize the pathogenicity of VUS, an optimal communication with treating physicians is required to obtain updated clinical information and/or tumor sample, as shown by the study of Burnichon et al. in which the re-examination and review of family history led to the classification of *NF1* germline variants as pathogenic[74]. In other cases, the knowledge of the catecholamine phenotype can help to assess the pathogenicity of the genetic variant found[88].

Current knowledge suggests that mutations in driver genes in PPGL are mutually exclusive. Thus, multiplexing different genes in parallel in TGPs aids VUS classification[94], as shown by the finding of a *NF1* somatic mutation (ID166) in a case in which we simultaneously found a germline VUS in *MEN1* (c.-10G>A), suggesting the latter is not pathogenic.

Other VUS could be more challenging to classify, as shown previously with co-occurring *NF1*[74] and *EPAS1*[98,110] variants. In our series, one patient harbored a double somatic mutation in *NF1* (ID434) and 2 cases double *EPAS1* variants (ID275: p.Pro531Thr and p.Leu400Pro; ID322: p.Asp539His and p.Gly537Gly). It is worthy to note that we only studied exon 9 of *EPAS1* in part II, but it seems that variants in exon 9 may be acting as modifier rather than causative of PPGL[110,181]. It was not possible to rule out that these second variants were acting as modifiers through appropriated functional assays, as previously performed with other *EPAS1*[92] or *MAX*[187].

In conclusion, VUS classification is a resource- and time-demanding task, and an international cooperative effort is required to update existing databases[188].

5.3 GENETIC STUDY AND CLINICAL DATA

5.3.1 GUIDED GENETIC STUDY USING CLINICAL DATA (PART I)

In part I, similarly to the COMETE cohort study, where somatic genetic assessment was guided by findings from genome-wide expression studies[73,74], our somatic study was, in part, guided by the fractionated biochemical profile observed for each tumor, highlighting the importance of having access to secretion data.

In addition to the predominant secretion, our study highlights the utility of differentiating tumor location to select not only the most appropriate DNA sample (germline or tumor), but also the

genes to be studied. Our analysis enabled us to conclude that the study of germline DNA should be prioritized in single HN-PGLs and T-PGLs, while the study of tumor DNA should be recommended in patients with single PCCs. Despite not finding statistically significant differences between the frequencies of somatic and germline mutations in A-PGLs, *SDH* genes were involved in 72% of mutated cases (being only two somatic mutations). In addition, 20% (3/15) of *HRAS*-mutated cases in this series were A-PGLs, compared with the 4.2% (1/24) previously reported[79,95,117], highlighting the relevance of *HRAS* somatic testing in tumors located outside the adrenal glands. Consequently, for A-PGLs, it seems appropriate to recommend a germline study (starting with the *SDH* genes) in cases with tumors negative or without SDHB-IHC and somatic screening (excluding the *SDH* genes) in those with positive SDHB-IHC staining.

5.3.2 "BLINDED" GENETIC STUDY USING TGPs (PART II)

TGPs genetic results made evident that some pitfalls could occur relying too much in clinical data to guide genetic testing.

Inevitably, the mutation detection rate in part II is dependent on the extent of previous conventional genetic screening using algorithms based on available clinical data. In a study by Rattenberry et al. NGS was shown to successfully detect mutations in previously unstudied cases[94]; our data additionally demonstrates that TGPs can detect mutations in genes that have been previously disregarded due to discordant or missing clinical data. Driver mutations were found in 37 of the 289 Wt^{ps} (13%): germline mutations 13/289 (4.5%), and somatic mutations 24 of the 83 WT^{ps} tumors (29%). This finding highlights the risk of relying excessively on phenotypic features to guide mutation testing.

For instance, two patients older than 60 years with a single PCC were found to be carriers of a germline mutation in *VHL* (ID374; p.Arg200Trp) or *RET* (ID283; p.Phe776Leu). These mutations would probably had been overlooked if methods other than TGPs had been applied. These results are crucial for the management of both index cases and their relatives, as theses specific mutations have been related to polycythemia[189] and MTC[190], respectively. This approach also allowed us to detect *NF1* somatic mutation in 2 TA-PGL cases, despite this gene being mainly associated with PCCs[73,176].

Another confounding factor could be the biochemical secretion. In this regard, these data will help to guide screening, but there are incongruous values due to variation in sample collection procedures or interfering drugs or foods[118]. Of note, in this study 1 *VHL* and 1 *FH* mutation were detected in adrenergic-secreting tumors, and 3 *RET*-mutated cases were noradrenergic. Furthermore, 9 cases with a *NF1* somatic mutations presented noradrenergic secretion. While

NF1 has been classically associated with an adrenergic secretion, the heterogeneous profile of *NF1* tumors had been pointed out before[75].

A remarkable finding was a *NF1* somatic mutation in a patient with multiple PCC (ID357). After reviewing the pathological report, the tumor was reclassified as a single multi-lobulated PCC.

5.4 AGE AT ONSET

Although there is no agreement on the upper age limit to apply for genetic testing[8,11,36–38,42,191], 45 years resulted in a better identification of mutation-positive cases in the study of Erlic et al., where various clinical parameters were assessed using multiple logistic regression[192].

Taking into account this limit of age in part I (**Figure 5**), we analyzed the possible consequences of limiting genetic studies depending on the age at presentation.

Thus, if germline screening had not been performed in index cases older than 45 years in our series, we would have missed 11.1% hereditary cases of HN-PGLs, 25% of T-PGLs, 8% of A-PGLs and 3.2% of PCCs. Conversely, younger patients tend to be excluded from somatic studies. Thus, if the somatic screening had not been performed in index cases younger than 45 years, we would have missed the genetic diagnosis of 42.9% A-PGLs, and even more importantly, 53.3% of PCCs.

Therefore, we recommend that a germline and somatic genetic diagnosis be carried out for all S-PPGLs, regardless of the age of diagnosis.

Historically, pediatric age has been considered a predictor of the presence of germline mutations in PPGL-related genes. In fact, previous pediatric series have shown rates of germline mutation of around 80%[41]. The results from part I in bona fide cases showed that at least half of pediatric S-PPGL presented a germline mutation, and a quarter of S-PPGLs could be explained by a somatic mutation.

In part II, 12 out 13 pediatric patients included in the study show enough amplified to be evaluated, being able to detect a germline mutation in 25% and a somatic mutation in 29%. Among them, 7 (58%) had been previously studied using Sanger, and a somatic mutation in *VHL* (ID153) and a gross deletion in *SDHB* (ID152) could be detected.

In conclusion, both parts of this thesis highlight that it is also important to study somatic mutations in young patients, being *VHL* the main player (100% of somatic were located in this gene in both parts of this thesis). On the other hand, *SDHB* represented 75% and 100% of the germline mutations found in pediatric patients in part I and part II, supporting that this gene

should be the first one to be studied if NGS is not available, not only for the prevalence, but also for the metastatic rate associated to this gene[4,15–17].

Moreover, pediatric cases with somatic mutations should be assessed with caution, as it is especially important to rule out the presence of mosaicism. In this regard, NGS has been proven useful as a diagnostic tool to accurately quantify the level of mosaicism through the study of different embryological lineage cells[180]. To note, *VHL* somatic mutations in part II were detected using TGPs in a frequency of reads of the altered variant around 28% and 29% in tumor DNA from patient ID190 and ID153, respectively, which could be an indicator of the presence of mosaicism, among other factors previously mentioned (e.g. normal tissue contamination). Thus, if a somatic mutation is found in a pediatric case, a search for the mutation in multiple tissues is encouraged to better evaluate the extension of the disease, as well as to improve the management and follow-up of the patient and their offspring[180].

5.5 METASTATIC BEHAVIOUR

As expected, *SDHB* was the main gene involved in metastatic S-PPGLs, even in cases with PCC (40% of metastatic PCC with a driver mutations identified). It was the most commonly mutated gene among metastatic T-PGLs (80%). Somatic mutations in *VHL*, *RET*, *EPAS1*, *HRAS* and *SDHD* were detected in one case each. Thus, because knowing the driver mutation is especially important in the determination of the most appropriate therapeutic intervention[35], metastatic cases should not be excluded from comprehensive testing for somatic mutations.

NGS allowed us to detect mutations in *SDHA*, *VHL*, *NF1* and *HRAS* among metastatic cases in which *SDHB* involvement had been ruled out, as 20 (67%) had been previously studied using Sanger sequencing. These genes would likely had been ignored and the diagnosis delayed due to the low prevalence of metastatic cases reported with mutations in these genes, as well as the large size of some of them. Of note, we found the second malignant case related to a *HRAS* mutation (ID376).

5.6 SINGULAR FEATURES IN CLINICAL DATA

Surprisingly, despite black PPGL being rare, the two cases in our series were related to *RET* variants. Patient ID164 has been reported[193] and case ID429 harbored a germline synonymous *RET* variant in exon 11 previously demonstrated to alter the splicing of the gene in HD[173]. The co-occurrence of MEN2 and HD is intriguing, since the latter is caused by *RET* inactivating mutations, and MEN2 to activating ones. However, MTC incidence among HD patients varies between 2.5 and 5%, with all activating mutations located in exon 10[194]. As it was not possible

to perform functional studies to assess the pathogenicity of this specific variant, it was classified as a VUS and the recommendation for this case would be to follow it as a potential MEN2 case.

5.7 SEQUENCING APPROACHES

Nowadays TGPs are broadly used due to its cost-effectiveness and ease of management. Several groups have already used this technology for PPGL genetic testing[94,98,195,196]. While it is difficult to compare these studies, mainly due to their different design[196], it is clear that an optimal and uniform multiplexing of all regions of interest is yet to be established.

Rattenberry et al. suggested near equal quality of TGPs to Sanger sequencing in PPGL, and a significant reduction in both cost and time consumption[94]. Similar performance of diagnostic TGPs has been reported by an accumulating number of observations in other diseases using different enrichment assays and sequencing platforms[94,197–200]. However, current guidelines for the diagnostic use of NGS state that the validity of the selected bioinformatic software needs to be ensured by the local investigator before clinical application[201]. Thus, the local laboratory should select, validate and maintain a robust bioinformatics pipeline, a process that will require trained and experienced personnel. These investments and the running costs of bioinformatic processing will inevitably increase cost of TGPs[202].

The momentum of NGS in a clinical setting was recently strengthened by demonstrating equal quality of generated results compared to Sanger sequencing[203]. In a study of Crona et al. in PPGL tumor samples, Targeted NGS was performed using Truseq custom amplicon enrichment sequenced with a double strand design (such as panel II of part II of this Thesis) on an Illumina MiSeq instrument. Results were analysed in parallel using 3 bioinformatics pipelines (Commercially available MiSeq Reporter 2.1.43 (MSR), CLC GenomicsWorkbench 5.51 (CLC) and the in-house custom pipeline (ICP), and compared to results from traditional Sanger sequencing. Compared to Sanger sequencing, variant calling revealed a sensitivity ranging from 83 to 100% and a specificity of 99.9-100%, demonstrating that TGPs show equal performance and comparable quality to Sanger Sequencing in PPGL. To note, only MiSeq reporter identified all pathogenic variants in both sequencing runs detected by Sanger Sequencing[204], this is the main reason why we used it in part II.

Herein, we designed a comprehensive TGPs for PPGL, including for the first time *EGLN1/PHD2*, *EGLN2/PHD1, MEN1* and *MDH2*, and screened a large international multicenter series of patients using germline and tumor DNA. In addition, we performed a stringent process of validation and a multi-step workflow analysis to confirm this platform as an efficient and accurate alternative to conventional sequencing in the diagnosis setting. We used MiSeq

reporter, and our pipeline allowed us to rescue pool biased variants, as well as indels such as the *NF1* frameshift variant (c.7269_7270delCA) in ID445, as troubles regarding these types of variants had been previously reported with Illumina's platform[98,204]. Consistent with previous reports[94,98,195,196], the sensitivity of the TGPs was extremely high (99.5%).

As this platform is not able to multiplex all the regions designed with a well coverage and it is not able to detect gross deletions, the workflow of this study included in a second step the conventional sequencing of the regions with low coverage and the study of gross deletions. In this regard, even applying a stringent threshold of 50-fold coverage, we did not find any additional variants in the Sanger sequencing of these regions, suggesting that the 30-fold coverage threshold used in the study of Rattenberry et al. is appropriated[94]. The MLPA/Multiplex analysis of TGPs negative patients diagnosed an additional case (0.3%, 1/291 germline DNA available), highlighting that gross deletions are rare events (<1%)[35]. Further, performing an MLPA/Multiplex study on selected cases as a second step reduces cost and processing time, and the protocol can be even more focused using SDHB, 2SC, and MAX- IHC[54,60,177].

In comparison with previous TGPs studies, we used the variant filtering threshold to prioritize variants for validation, instead of using it for filtering them out. Applying fixed thresholds can significantly reduce the detection sensitivity for heterozygous variants due to normal tissue contamination[30], intra-tumor heterogeneity[139] and mosaicism[47,81–83]. Three cases showed potential mosaicism, as the variants were detected in around 20% of reads, 2 affecting *VHL* in pediatric cases previously mentioned (ID153 and ID190), and 1 involving *SDHD* (ID296), the latter not previously described.

5.8 DNA SAMPLES

5.8.1 FFPE TUMOR SAMPLES

The prevalence of mutations in the *SDH* genes in A-PGLs, metastatic cases, as well as pediatric S-PPGLs cases stresses the importance of using SDHB-IHC as a filter to optimize genetic screening in part I, and therefore highlights the importance of having access to FFPE tumor material[35,137]. A good example of utility of performing IHC to guide the genetic study using Sanger sequencing was the case with a somatic mutation in *SDHD,* as *SDH* genes mutations are scarce and rarely analyzed.

When FFPE tumor material is unavailable, at a minimum *SDHB* germline mutations should be tested for, given the higher associated risk of developing metastases[15], and the presence of a founder effect, at least in the Spanish population[37].

In part II, FFPE tumor samples to perform IHC studies was used in the filtering process to select the genetic variants that should be validated by Sanger sequencing, but also to test the pathogenicity of VUS found in these genes[94,95,97].

5.8.2 SOURCE AND QUALITY OF DNA SAMPLES

Frozen and blood DNA samples have optimal quality for molecular diagnosis. However, their use is not always feasible, as saliva, GenomiPhi or FFPE tumor samples are sometimes the only available DNA source.

In part I we used germline DNA derived from blood and tumor DNA from FFPE and frozen samples. In part II, we tried to analyze all the patients with the available DNA source, independently of the type of sample. Saliva DNA samples performed well, as the germline mosaicism in *SDHD* was detected in a similar percentage of reads to that in the blood DNA of the same patient (ID296). Additionally, samples amplified by GenomiPhi were found to be useful for diagnostic purpose as our panel detected all SNPs previously identified by Sanger sequencing.

A common problem with FFPE samples is the high number of false-positive variants resulting from deamination (C:G>T:A); this was the main reason why *NF1* was not tested in FFPE samples in part I of this thesis. In part II, this circumstance was resolved by applying doubled stranded-TGP. In addition, the use of Covaris system in part II improved the DNA extraction efficiency and the percentage of cases diagnosed, in comparison with part I in which we used Qiagen extraction, since the FFPE samples in which DNA was extracted with the Covaris system showed a higher number of reads/amplicon. In fact, the amplification failed in less FFPE samples than in blood or frozen tissue DNA. Thus, in part II we were able to study all types of DNA samples with similar performance.

We therefore consider critical the access to the tumor sample for a complete PPGL genetic screening and diagnosis. The study of DNA from tumor sample as the first step allows “to kill three birds with one stone”, as allows the detection of germline, somatic and mosaic mutations.

In our series, the frequency of somatic mutations (43.4% and 32%, in part I and II, respectively) was in agreement with previous reports, even in cases highly likely to carry a germline mutation. Thus, a somatic was found in 60% and 28.6% of pediatric cases, 29% and 19% of malignant in part I and II, respectively, and 7% of multiple tumor cases studied in part II. Furthermore, studying the tumor DNA of apparently familial cases can reveal phenocopies.

5.9 FUTURE OUTCOMES

Despite using TGPs, a comprehensive clinical record is still useful when performing genetic diagnosis, as demonstrated by findings for case ID79. This patient was diagnosed with a GIST and multiple noradrenergic PGLs. The tumor showed negative SDHB-IHC, TGPs did not detect any *SDH* variant and gross deletions were also ruled out. Our workflow allowed us to select this case to be further studied using multi-omics platforms, to finally detect a functional epi-mutation in *SDHC,* which is an event recently described as causing the disease [104].

As the list of new PPGL genes is growing constantly, their inclusion to already designed panels is not a cost-effective process, as it requires the generation of new libraries and their validation. Our workflow allowed us to select the specific cases that would benefit from further genetic screening. Examples of this point are the implementation of the study of *MERTK*[58] and exon 7 of *RET*[163] in patients with PCC and MTC, despite no mutation being found, or the selection of WT composite tumors to further study *ATRX* (35 exons), which has not only been related to composite PCC, but also to metastatic PPGL[106].

5.10 REASONS TO CONSIDER GENETIC SCREENING IN ALL PPGL CASES

Genetic screening is expensive and time-consuming, especially if NGS is not available, but there are important implications of having a genetic diagnosis in S-PPGLs. According to the American Society of Clinical Oncology (ASCO)'s general recommendations for genetic screening, all patients with a risk of at least 10% of carrying a genetic mutation should be offered genetic testing, especially when the results would aid in diagnosis or influence the management of the patient or family members at hereditary risk of cancer[116,205].

The identification of germline or mosaic mutations allow the early diagnosis of multiple tumors or additional syndromic neoplasias in the proband, as well as in relatives at risk. On the other hand, the identification of a somatic mutation benefits: (1) family screening, as it frees relatives from the need for genetic screening and clinical follow-up (more caution has to be taken in the case of 'somatic mutations' in pediatric cases since the possibility of a germline mosaicism cannot be excluded); (2) diagnosis, making unjustified the exhaustive follow-up required for patients harboring germline mutations associated with a high risk of developing multiple tumors and different cancer types; (3) prognosis, as it is known that mutations in some genes have a well-known high risk of metastatic behavior, and; (4) therapeutic opportunities, since the identification of the mutated gene and the corresponding pathway opens up the possibility of new therapeutic approaches if surgery is not curative. Regarding this latter point, it has been proposed that mutations involving cluster 1 genes could be targeted using an antiangiogenic approach, mutations in cluster 2 genes could be treated by targeting the mTOR and the RAS–

RAF pathway and, specifically, for *FH*-related and *SDH*-related malignant PPGLs, drugs targeting epigenetic pathways could be an option[1,24,35,84].

Finally, the identification of a somatic mutation avoids additional germline genetic screening as new susceptibility genes are discovered, which is associated with considerable anxiety and psychological ill health, especially in relatives of pediatric and metastatic cases[33,35,97,206,207].

In conclusion, the results of this study should bring to an end years of controversy and debate, as it brings new evidence that highlights the need to recommend genetic testing for all patients with PPGL, regardless of the apparent sporadic presentation, or the age at first PPGL diagnosis.

In summary, Sanger sequencing of the appropriated gene in syndromic cases, as well as *SDHB* in pediatric, multiple and metastatic cases is still an effective first step approach, with TGPs as the most reasonable second step. In the case of S-PPGL, for laboratories where TGPs is not available or not optimized, we propose a genetic testing algorithm based on tumor location for sporadic single PPGL based on the present and previous findings[5,35,94–99,112,116,208] (**Figure 13**). Where NGS can be used, the sample type that should be tested is tumor DNA for PCCs and germline DNA for HN-PGLs and T-PGLs. For A-PGLs, it seems crucial to have a FFPE tumor sample available in order to perform SDHB-IHC, the findings from which can be used to determine the ideal source of DNA sample to study.

On the other hand, before applying TGPs in clinical setting, it is critical to ensure: adequate library preparation; high accuracy; and avoidance of false positive and negative results through the implementation of alternative techniques. Thus, this technology should be performed in specialized and accredited laboratories with expertise in PPGL[8].

Here, we have demonstrated the effectiveness and feasibility of this diagnostic tool, able to detect low-coverage, pool biased and indel variants. We conclude that our TGPs workflow enables the study of the main driver PPGL genes in different DNA sources, and improves the clinical management of index cases and their relatives at risk. In addition, TGPs is the optimal method to select cases that will benefit from further investigation in a research setting, as the etiology of one third of PPGL cases remains in the darkness.

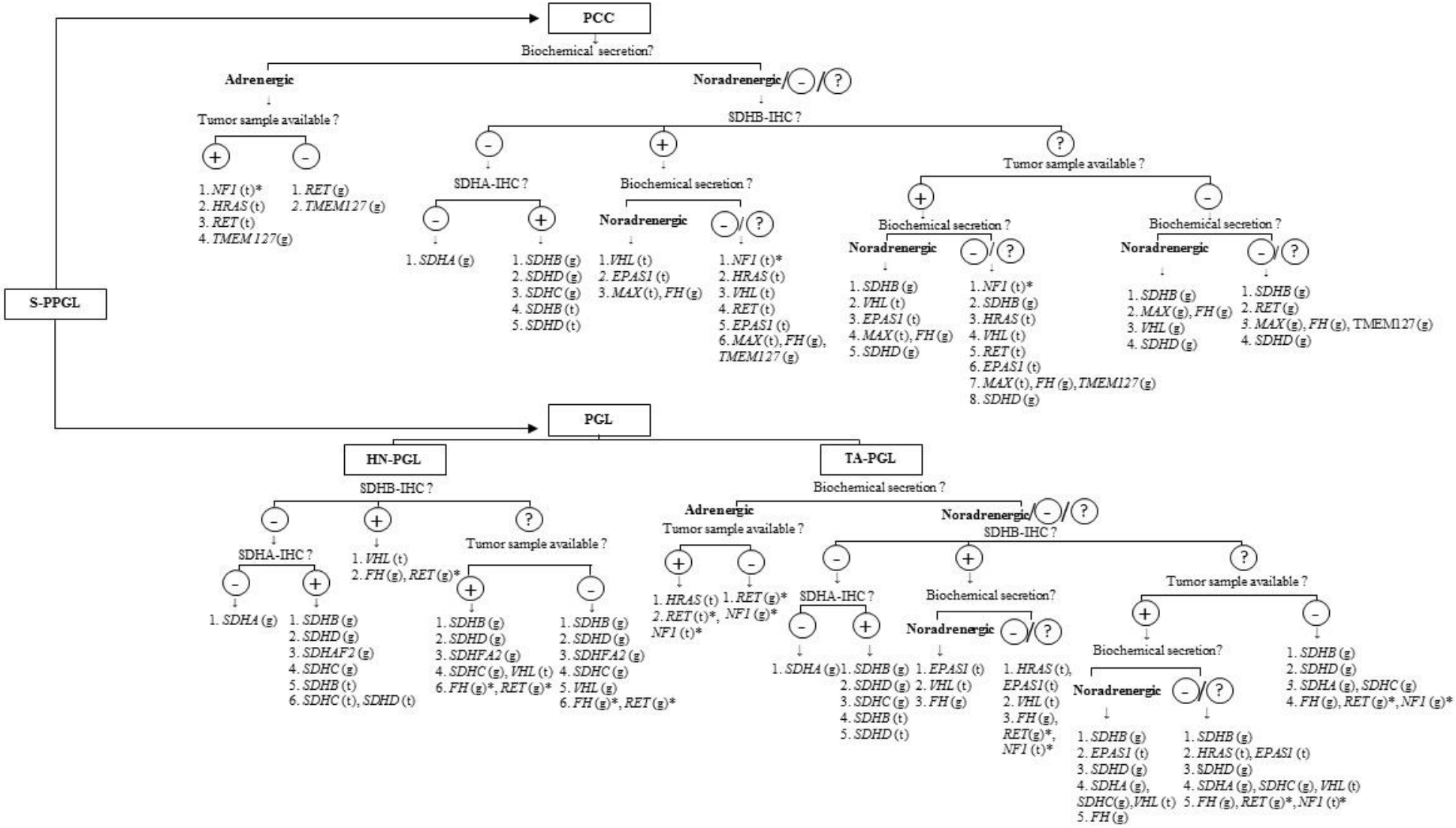


Figure 13. Proposed genetic testing algorithm for patients with sporadic-pheochromocytoma and paraganglioma (S-PPGL) based on SDHB-immunohistochemistry (IHC) in formalin-fixed paraffin-embedded tissue (if available) and biochemical phenotype.

This algorithm has been elaborated considering previous reports' findings[5,35,94–99,112,116,208] and the current series. (g), germline DNA; HN-PGL, head and neck-paraganglioma; PCC, pheochromocytoma; PGL, paraganglioma; (t), tumour DNA; TA-PGL, thoracic plus abdominal-paraganglioma. *Test if possible.

VI. CONCLUSIONS

6.1 The results of this study bring new evidence regarding the need to recommend genetic testing for all patients with PPGL, regardless of the apparent sporadic presentation, or the age at diagnosis.

6.2 It is also important to study somatic mutations in young patients, metastatic, and multiple cases. Furthermore, studying the tumor DNA of apparently familial cases can reveal phenocopies. Identifying a somatic mutation not only benefits family screening, diagnosis, prognosis, therapeutic opportunities, but also avoids additional germline genetic screening as new susceptibility genes are discovered, which is associated with considerable anxiety and psychological ill health.

6.3 In S-PPGL, in addition to the predominant secretion and SDHB-IHC staining, our study highlights the utility of differentiating tumor location to select not only the most appropriate DNA sample (germline or tumor), but also the genes to be studied. For laboratories where TGPs are not available or not optimized, we have proposed a genetic testing algorithm using Sanger sequencing. Where NGS is available, the study of germline DNA should be prioritized in HN-PGLs and T-PGLs, while the study of tumor DNA should be recommended in PCCs. For A-PGLs, it seems appropriate to recommend germline study (starting with the *SDH* genes) in cases with tumors negative or without SDHB-IHC, and somatic screening (excluding the *SDH* genes) in those with positive SDHB-IHC staining.

6.4 Sanger sequencing of the appropriated gene in syndromic cases, as well as *SDHB* in pediatric, multiple and metastatic cases is still an effective first step approach, being TGPs the most reasonable second step in the genetic diagnosis of PPGL.

6.5 We have demonstrated the effectiveness and feasibility of two TGPs as diagnostic tools in the clinical setting, able to detect low-coverage, pool biased and indel variants. In addition, our TGPs-workflow enables the study of the main driver PPGL genes in different DNA sources with similar performance, and improves the clinical management of index cases and their relatives at risk. Furthermore, TGPs are the optimal methods to select cases that will benefit from further investigation in a research setting, as the etiology of one third of PPGL cases remains unknown.

6.6 The access to the tumor sample is critical for a complete PPGL genetic screening and diagnosis. The study of tumor DNA as the first step allows “to kill three birds with one stone”, as allows the detection of germline, somatic and mosaic mutations. To note, FFPE tumor sample is very useful, as SDHB-IHC is not only used to guide the genetic study using Sanger

sequencing in S-PPGL, but also in TGPs in the filtering process, as well as to test the pathogenicity of VUS.

VI. CONCLUSIONES

6.1 Los resultados de este estudio aportan nuevas evidencias sobre la necesidad de recomendar el estudio genético en todos los pacientes con feocromocitomas y paragangliomas (FPGL), independientemente de si la presentación es aparentemente esporádica (FPGL-E) o la edad en el momento del diagnóstico.

6.2 Es importante estudiar la presencia de mutaciones somáticas en los pacientes jóvenes y/o con tumores metastásicos y/o múltiples. Además, el estudio del ADN tumoral de casos aparentemente familiares puede revelar la presencia de fenocopias. La identificación de una mutación somática, no sólo beneficia el consejo familiar, el diagnóstico, seguimiento y el planteamiento de las posible opciones terapéuticas, sino que también evita continuar insistiendo en el estudios genéticos del ADN germinal a medida que se descubren nuevos genes de susceptibilidad, que se asocia a la presencia de ansiedad considerable e inestabilidad emocional.

6.3 Nuestro estudio pone de relieve que en los FPGL-E, además de la secreción predominante y el resultado de la inmunohistoquímica de SDHB (IHC-SDHB), es útil tener en cuenta la localización del tumor primario no sólo para determinar la muestra de ADN más apropiada (germinal o tumoral), sino también los genes a estudiar. En los laboratorios en los que los paneles de genes no están disponibles o no se han puesto a punto, hemos propuesto un algoritmo de diagnóstico genético utilizando la secuenciación por Sanger. En los laboratorios en los que los paneles de genes están disponibles, el estudio del ADN germinal debe ser priorizado en el caso de paragangliomas de cabeza, cuello y torácicos, y el estudio del ADN tumoral en el caso de feocromocitomas. En el caso de paragangliomas abdominales, recomendamos el estudio del ADN germinal (empezando por los genes *SDH*) en caso de tumores con el resultado de la IHC-SDHB negativo o no disponible, y el estudio del ADN tumoral (excluyendo el estudio de los genes *SDH*) en los casos con IHC-SDHB positiva.

6.4 El primer paso más eficaz en el diagnóstico genético es el estudio mediante secuenciación por Sanger del gen apropiado en los casos sindrómicos, así como el de *SDHB* en los pacientes pediátricos y en aquellos con tumores múltiples y/o metastásicos, siendo los paneles de genes el segundo paso más razonable.

6.5 Hemos demostrado la eficacia y viabilidad de dos paneles de genes como una herramienta de diagnóstico genético útil en la práctica clínica, capaces de detectar variantes con baja cobertura, variantes con sesgo de cobertura entre los dos diseños de amplicones y variantes de inserción y/o deleción. Además, nuestro algoritmo de trabajo basado en el uso de los

paneles de genes permite el estudio de los principales genes de FPGL en ADN de distinto origen con un rendimiento similar y mejora el manejo clínico de los casos índice y sus familiares. Además, los paneles de genes son el método óptimo para seleccionar los casos que se beneficiarán de ser incluidos en proyectos de investigación, dado que la causa de un tercio de los FPGL aún es desconocida.

6.6 La accesibilidad a la muestra tumoral es fundamental para completar el cribado genético y diagnóstico de los FPGL. El estudio primario del ADN tumoral permite "matar tres pájaros de un tiro", ya que permite la detección de mutaciones germinales, somáticas y de mosaico. Lo óptimo es disponer de la muestra tumoral parafinada. El resultado de la IHC-SDHB se puede utilizar no sólo para orientar el estudio genético mediante secuenciación por Sanger en FPGL esporádicos, sino también cuando se utilizan paneles de genes durante el proceso de filtrado de las variantes y para estudiar la patogenicidad de las variantes de significado desconocido encontradas.

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VIII. APPENDIX I: SUPPLEMENTAL MATERIAL

CUESTIONARIO CLÍNICO

REMITENTE

Hospital:

Médico responsable: **Servicio:**

Email de contacto: **Teléfono contacto:**

Fecha registro (día/mes/año):

PACIENTE

Nombre y apellidos:

Fecha nacimiento (día/mes/año):

Sexo: Hombre ☐ Mujer ☐ **Lugar de nacimiento:**

Etnia: Caucásica ☐ Africana ☐ Oriental ☐ Sud-Americana ☐

Peso: ☐ kg **Talla:** ☐ cm (en la cita en la que se firma consentimiento)

ANTECEDENTES PERSONALES

Sospecha diagnóstica inicial: Incidentaloma ☐ Síntomas ☐ Screening* ☐

*Screening por diagnóstico de CMT, por ser portador de mutación

Diabetes mellitus: Si ☐ No ☐; **Año de diagnóstico de DM:** ☐

Tensión arterial (TA):

1. Normotensión ☐ Hipotensión ☐ HTA ☐
2. En caso de HTA: persistente ☐ paroxística ☐ persistente con paroxismos ☐
3. Año de diagnóstico de HTA: ☐
4. TA en momento consentimiento(mmHg): Sistólica ☐ Diastólica ☐

Otros síntomas de presentación: Marcar en caso afirmativo:

Palpitaciones ☐ Cefalea ☐ Sudoración ☐ Dolor abdominal ☐ Dolor lumbar ☐

Otro:

Enfermedad genética o síndrome: En caso afirmativo, indicar cuál:

MEN 2 ☐ VHL ☐ NF 1 ☐ FEO/PGL familiar ☐ Otro:

Mutación:

Historia de tumores diferentes a feocromocitoma o paraganglioma: Si ☐ No ☐

Año de diagnóstico, localización y tipo:

ANTECEDENTES FAMILIARES

En caso afirmativo: Edad de diagnóstico, número, tipo y localización

- FEO/PGL:
- Otro tumor diferente a FEO/PGL:

DIAGNÓSTICO

Año de diagnóstico del primer feocromocitoma/paraganglioma:

Número de tumores: ☐ **Metástasis:** Si ☐ No ☐

Múltiples tumores primarios: Si ☐ No ☐

SI 1 TUMOR O EL QUE PROPICIA EL DIAGNÓSTICO:

LOCALIZACIÓN:

Adrenal izda ☐ dcha ☐ Torácica ☐ Abdominal ☐ Cervical ☐ cuerpo carotídeo ☐ glomus timpánico ☐ glomus supraaórtico ☐ glomus yugular/vagal ☐ Otra:

Comentario:

TAMAÑO (mm):

BIOPSIA: Si ☐ No ☐ FECHA (día/mes/año):

EMBOLIZACIÓN PREVIA: Si ☐ No ☐ FECHA (día/mes/año):

CIRUGÍA: Si ☐ No ☐ FECHA (día/mes/año):

Vía: Abierta ☐ Laparoscópica ☐ Reconversión* ☐

Descripción cirugía:

- Invasión loco-regional: Si ☐ No ☐

- Múltiples primarios: Si ☐ No ☐ En caso de respuesta afirmativa: Rellenar apartado Si > 1 tumor.

- Metástasis: Si ☐ No ☐ En caso de respuesta afirmativa: Localización y número:

- Resultado: Resección completa ☐ Tejido residual ☐

- En caso de tejido residual, especificar: Microscópico ☐ Macroscópico ☐

- Comentario:

Complicaciones intra-operatorias: Si ☐ No ☐ En caso de respuesta afirmativa:

Hipotensión ☐ Crisis HTA ☐ Hipoglucemia ☐ Arritmia ☐ Otra:

Complicaciones post-operatorias:

INFORME ANATOMO-PATOLÓGICO:

Diámetro máximo: X ☐ Y ☐ Z ☐ mm

Ki67 (%): Índice de proliferación:

Número de mitosis por 10 campos de gran aumento: Número de células contadas:

Necrosis: Si ☐ No ☐

Invasión: Capsular: Si ☐ No ☐ Adiposa: Si ☐ No ☐

Vascular: Si ☐ No ☐ Órganos adyacentes: Si ☐ No ☐

SI > 1 TUMOR:

Método diagnóstico:

Fecha diagnóstico:

LOCALIZACIÓN:

Adrenal izda ☐ dcha ☐ Torácica ☐ Abdominal ☐ Cervical ☐ glomus carotídeo ☐ glomus timpánico ☐ glomus supraaórtico ☐ glomus yugular/vagal ☐ Otra:

TAMAÑO (cm):

BIOPSIA: Si ☐ No ☐ FECHA (día/mes/año):

EMBOLIZACIÓN PREVIA: Si ☐ No ☐ FECHA (día/mes/año):

CIRUGÍA: Si ☐ No ☐ FECHA (día/mes/año):

Vía: Abierta ☐ Laparoscópica ☐ Reconversión* ☐

Descripción cirugía:

- Invasión loco-regional: Si ☐ No ☐

- Múltiples primarios: Si ☐ No ☐ En caso de respuesta afirmativa: Localización:

- Metástasis: Si ☐ No ☐ En caso de respuesta afirmativa: Localización:

- Resultado: Resección completa ☐ Tejido residual ☐

- En caso de tejido residual, especificar: Microscópico ☐ Macroscópico ☐

- Comentario:

Medicación utilizada para la preparación pre-quirúrgica:

Complicaciones intra-operatorias: Si ☐ No ☐ En caso de respuesta afirmativa:

Hipotensión ☐ Crisis HTA ☐ Hipoglucemia ☐ Arritmia ☐ Otra:

Complicaciones post-operatorias/secuelas:

INFORME ANATOMO-PATOLÓGICO:

Diámetro máximo: X ☐ Y ☐ Z ☐ mm

Ki67 (%): Índice de proliferación:

Número de mitosis por 10 campos de gran aumento: Número de células contadas:

Necrosis: Si ☐ No ☐

Invasión: Capsular: Si ☐ No ☐ Adiposa: Si ☐ No ☐

Vascular: Si ☐ No ☐ Órganos adyacentes: Si ☐ No ☐

DIAGNÓSTICO BIOQUÍMICO

PLASMA

Unidades plasmáticas: pg/mL ☐ ng/L ☐ nmol/L ☐

Fecha extracción (día/mes/año):			
Adrenalina plasmática:			
Noradrenalina plasmática:			
Dopamina plasmática:			
Catecolaminas plasmáticas: totales ☐ fraccionadas ☐			

Metanefrina libre plasmática:			
Normetanefrina libre plasmática:			
Metoxitiramina libre plasmática:			
Metanefrinas plasmáticas: totales ☐ fraccionadas ☐			
Cromogranina A suero:			

ORINA

24h ☐ Muestra aislada ☐

Unidades urinarias: µg/día ☐ mg/día ☐ µmol/día ☐

Fecha extracción (día/mes/año):			
Adrenalina libre urinaria:			
Noradrenalina libre urinaria:			
Dopamina urinaria:			
Catecolaminas totales urinarias: totales fraccionadas			
Ácido vanilmandélico/Ácido homovalínico:			
Metanefrina urinaria:			
Normetanefrina urinaria:			
Metanefrinas urinarias: totales fraccionadas			

OTRAS DETERMINACIONES REALIZADAS (Indicar unidades y rango)

- Cortisol ACTH
- Andrógenos
- Calcitonina
- Ca plasmático Fósforo plasmático Calciuria
  PTH vitamina D
- Hematíes hemoglobina EPO

DIAGNÓSTICO DE IMAGEN/EXTENSIÓN

☐ **Ecografía:** Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

☐ **TAC:** Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

☐ **RMN:** Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

☐ **MIBG:** Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

☐ **Octreoscan:** Positivo ☐ Negativo ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

☐ **FDG-PET:** Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐
Fecha (día/mes/año):

☐ **18F-DOPA-PET:** Positiva ☐ Negativa ☐ Metástasis: Si ☐ No ☐

Fecha (día/mes/año):

☐ **Arteriografía:** Positiva ☐ Negativa ☐

Fecha (día/mes/año):

☐ **Ecocardiograma:** Positiva ☐ Negativa ☐ Fecha (día/mes/año):
☐ **Otra:** Positiva ☐ Negativa ☐ Fecha (día/mes/año):

TRATAMIENTOS NO QUIRÚRGICOS: Marcar en caso afirmativo

☐ **MIBG:** Dosis:

5. Ciclos recibidos:
6. Fecha (día/mes/año) inicio-final:

☐ **Quimioterapia:** Agente/s utilizados: Dosis:

7. Ciclos recibidos:
8. Fecha (día/mes/año) inicio-final:

☐ **Ablación por radiofrecuencia:**

9. Fecha (día/mes/año) inicio-final:
10. Localización:

☐ **Radioterapia externa:** Dosis:

11. Ciclos recibidos:
12. Fecha (día/mes/año) inicio-final:
13. Localización:

☐ **Radionúclidos:** Agente utilizado: Dosis:

- Ciclos recibidos:
- Fecha (día/mes/año) inicio-final:
- Localización:

☐ **Quimioembolización:** Agente utilizado:

14. Fecha (día/mes/año):

15. Localización:

☐ **Tratamiento molecular:** Agente utilizado: Dosis:

16. Fecha (día/mes/año) inicio-final:

SEGUIMIENTO

Fecha última revisión (día/mes/año):

Estado actual:

☐ Fallecimiento: Fecha (día/mes/año):

Causa:

☐ Vivo con enfermedad residual

☐ Vivo libre de enfermedad

Comentario:

MUESTRA REMITIDA

☐ TUMOR: Congelado ☐ En parafina ☐ DNA tisular ☐

Fecha extracción (día/mes/año):

☐ TEJIDO NORMAL: Congelado ☐ En parafina ☐ DNA tisular ☐

Fecha extracción (día/mes/año):

☐ SANGRE: ENTERA ☐ SUERO ☐ PLASMA ☐

En caso de plasma, especificar si: PLASMA EDTA ☐ PLASMA HEPARINIZADO ☐

Fecha extracción (día/mes/año):

☐ DNA leucocitos

Fecha extracción (día/mes/año):

Supplementary table S1. Clinical and genetic data from the 329 patients included in the study.

Nº	ID	Sex	Age	Tumor	Behaviour	Diagnosis	BC secretion	Tumor sample	SDHB-IHC	Mutation	Gene	Mutation	Date ressection primary tumor (or diagnosis if no ressection)	Last follow-up date	Last follow-up status	Met. location	Met. time	Met. diagnosis
1	3	M	59	HN-PGL	Benign	sympt. (local mass)	NS	Frozen and FFPE	ND	Germline	*SDHC Δ*	c.253_255dupTTT, p.Phe85dup	09/2003	04/2006	Alive, disease free			
2	62	M	64	PCC	Benign	sympt. (adrenergic)	No sec.	FFPE	Positive	Somatic	*HRAS*	c.37G>C, p.Gly13Arg	01/1995	02/2014	Alive, disease free			
3	78	M	11	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Not available	No			06/1999	02/2006	Alive, disease free			
4	91	F	52	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			12/2002	03/2014	Alive, disease free			
5	118†	F	64	PCC	Benign	inc. (surgery)	NS	FFPE	Positive	Somatic	*HRAS*	c.182A>G, p.Gln61Arg	01/2004 (necropsy)	01/2004	Deceased (necropsy after cardiovascular shock)			
6	130	M	74	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			10/2006	02/2014	Alive, disease free			
7	167	M	51	PCC	Benign	sympt. (adrenergic)	A	FFPE	Positive	Somatic	*HRAS*	c.181C>A, p.Gln61Lys	06/2002	01/2012	Alive, disease free			
8	178	F	22	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			03/1997	01/2014	Alive, disease free			
9	66†	F	19	A-PGL	Malignant	sympt. (adrenergic)	Nad.	FFPE	ND	Germline	*SDHB*-GD Δ	exon 1 gross deletion	04/1997	01/2002	Deceased (no response to chemotherapy)	Bone, liver and pituitary	Metac., 15 months after first	Image (MR, MIBG) and BC study.

																	surgery	
10	235	M	76	PCC	Benign	inc. (surgery)	No sec.	FFPE	Positive	No			01/2004	02/2014	Alive, disease free			
11	287	F	68	HN-PGL	Benign	sympt. (local mass)	NS	FFPE	Not available	No			02/2006	02/2006	Alive, post-surgery			
12	299	F	32	A-PGL	Malignant	sympt. (adrenergic)	Nad.	FFPE	Positive	No			04/2006	02/2013	Alive, disease free	Local lymph node	Sync.	AP study
13	335	F	62	A-PGL	Benign	inc. (image)	Nad.	FFPE	Positive	Somatic	*EPAS1*	c.1592C>T, p.Pro531Leu	04/2008	05/2011	Alive, disease free			
14	344	F	52	PCC	Malignant	NS	NS	FFPE	Positive	Somatic	*EPAS1*	c.1606C>A, p.Asp536Tyr	08/2001	05/2009	Alive, met.	Local lymph node	Metac., 36 months after first surgery	Image (MIBG)
15	379	F	51	A-PGL	Benign	inc. (surgery)	NS	FFPE	Positive	Somatic	*HRAS*	c.181C>A, p.Gln61Lys	04/2005	11/2009	Alive, disease free			
16	396	F	53	A-PGL	Benign	sympt. (local mass)	No sec.	FFPE	Positive	Somatic	*HRAS*	c.37G>C, p.Gly13Arg	05/2010	11/2010	Alive, disease free			
17	419	F	59	HN-PGL	Benign	NS	NS	FFPE	Positive	No			11/2006	11/2006	Alive, post-surgery			
18	450	F	51	HN-PGL	Benign	sympt. (adrenergic)	No sec.	FFPE	Positive	No			11/2011	05/2014	Alive, disease free			
19	452	F	50	A-PGL	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			01/2011	01/2013	Alive, disease free			
20	460	F	62	PCC	Malignant	sympt. (adrenergic)	A	FFPE	Positive	Somatic	*HRAS*	c.182A>G, p.Gln61Arg	05/2009	01/2013	Alive, disease free	Local lymph node	Sync.	AP study
21	103	M	70	PCC	Benign	NS	NS	Frozen	ND	Somatic	*RET*	c.1900T>C, p.Cys634Arg	01/2000	06/2003	Alive, disease free			

22	470	M	51	PCC	Benign	inc. (image)	Nad.	FFPE	Positive	No			11/2011	07/2012	Alive, disease free			
23	478	M	47	A-PGL	Benign	inc. (image)	Nad.	FFPE	Positive	No			06/2012	06/2012	Alive, post-surgery			
24	482	F	76	T-PGL	Benign	NS	NS	FFPE	Positive	No			06/2011	06/2014	Alive, disease free			
25	484	M	45	A-PGL	Benign	NS	Nad.	FFPE	Positive	No			07/2012	01/2013	Alive, disease free			
26	493	M	52	HN-PGL	Benign	sympt. (local mass)	No sec.	FFPE	Positive	No			10/2012	10/2012	Alive, post-surgery			
27	507	F	70	PCC	Malignant	sympt. (adrenergic)	NS	FFPE	Positive	No			08/2011	02/2013	Alive, met.	Bone and liver	Sync.	Image (CT, MR, Octreoscan) and BC study
28	508	F	62	HN-PGL	Malignant	sympt. (local mass)	Nad.	FFPE	Positive	No			01/2014	02/2014	Alive, post-surgery	Local lymph node	Sync.	AP study
29	512	F	46	PCC	Benign	inc. (image)	Nad.	FFPE	Positive	No			10/2009	01/2014	Alive, disease free			
30	242	M	25	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen and FFPE	Positive	Somatic	*VHL*	c.250G>C, p.Val84Leu	12/2002	01/2006	Alive, disease free			
31	587†	M	81	A-PGL	Benign	sympt. (adrenergic)	A	FFPE	Positive	Somatic	*HRAS*	c.181C>A, p.Gln61Lys	05/2007	01/2012	Deceased (heart attack, disease free)			
32	591	F	26	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			04/2007	02/2013	Alive, disease free			

33	594	F	45	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			08/2006	06/2013	Alive, disease free			
34	599	M	74	PCC	Benign	inc. (image)	Nad.	FFPE	Positive	No			02/2007	06/2012	Alive, disease free			
35	600	F	47	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			03/2007	11/2011	Alive, disease free			
36	612	M	56	PCC	Benign	inc. (image)	Nad.	FFPE	Positive	No			06/2007	02/2014	Alive, disease free			
37	621	M	29	PCC	Benign	inc. (image)	A	FFPE	Positive	No			04/2009	01/2014	Alive, disease free			
38	630	M	42	PCC	Benign	sympt. (adrenergic)	A	FFPE	Positive	No			04/2010	12/2012	Alive, disease free			
39	636	M	42	PCC	Benign	inc. (image)	NS	FFPE	Positive	Somatic	*HRAS*	c.182A>G, p.Gln61Arg	07/1996	07/2010	Alive, disease free			
40	637	F	9	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			10/2009	01/2013	Alive, disease free			
41	641	M	57	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			03/2008	04/20014	Alive, disease free			
42	157	M	30	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	Germline	*SDHB*-GD Δ	exon 1 gross deletion	02/2001	07/2001	Alive, disease free			
43	647	F	28	PCC	Benign	sympt. (adrenergic)	A	FFPE	Positive	Somatic	*HRAS*	c.182A>G, p.Gln61Arg	01/2001	11/2010	Alive, disease free			
44	649	M	14	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	Somatic	*VHL*	c.260T>C, p.Val87Ala	07/2011	03/2013	Alive, disease free			
45	653	M	NS	PCC	Benign	NS	NS	FFPE	Positive	Somatic	*RET*	c.2753T>C, p.Met918Thr	01/2013	01/2013	Alive, post-surgery			
46	657†	F	75	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	Somatic	*EPAS1*	c.1592C>T, p.Pro531Leu	11/1996	09/2013	Deceased (unknown cause, but			

															PPGL free			
47	175	F	36	T-PGL	Malignant	inc. (surgery)	Nad.	FFPE	ND	Germline	*SDHB Δ*	c.278G>A, p.Cys93Tyr	01/2001 (palliative surgery)	05/2013	Alive, met. (palliative surgery)	Bone and lung	Metac., 24 months after palliative surgery	Image (MR, MIBG) and BC study
48	658†	F	75	PCC	Benign	NS	NS	FFPE	Positive	Somatic	*HRAS ◊*	c.182A>G, p.Gln61Arg	01/1998	03/1999	Deceased (unknown)			
49	659	F	27	PCC	Benign	sympt. (adrenergic)	A	FFPE	Positive	Somatic	*HRAS*	c.182A>G, p.Gln61Arg	02/1999	01/2003	Alive, disease free			
50	886	M	57	PCC	Benign	inc. (image)	A	FFPE	Positive	No			02/2013	06/2013	Alive, disease free			
51	889	F	43	PCC	Benign	sympt. (adrenergic)	A	FFPE	Positive	Somatic	*RET*	c.2753T>C, p.Met918Thr	04/2013	02/2014	Alive, disease free			
52	890	F	49	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	No			01/2013	04/2013	Alive, disease free			
53	971	M	20	PCC	Malignant	sympt. (adrenergic)	Nad.	FFPE	Positive	No			09/2007	02/2013	Alive, disease free	Local lymph node, bona and liver	Sync. (local lymph node) and Metac. (bone and liver, 36 months after the first surgery)	Image (MR, MIBG, FDG-PET) and BC study
54	1004	F	45	A-PGL	Malignant	sympt. (local mass)	NS	FFPE	Negative and	Somatic	SDHD	c.112C>T, p.Arg38*	05/2010	06/2014	Alive, met.	Bone, lung	Sync. (bone)	Image (MR, MIBG,

									positive IHC SDHA							and liver	and Metac. (lung and liver)	Octreoscan) and BC study
55	1007	M	68	PCC	Benign	NS	NS	FFPE	Positive	No			01/2014	01/2014	Alive, post-surgery			
56	1010	M	42	A-PGL	Benign	NS	NS	FFPE	Positive	No			11/2013	07/2014	Alive, local residual disease			
57	465	F	15	A-PGL	Benign	sympt. (local mass)	Nad.	FFPE	Positive	Somatic	*VHL*	c.191G>C, p.Arg64Pro	07/2011	08/2011	Alive, post-surgery			
58	63	F	35	PCC	Benign	sympt. (adrenergic)	NS	Frozen	ND	Somatic	*RET*	c.2753T>C, p.Met918Thr	04/1999	04/1999	Alive, post-surgery			
59	133	F	38	PCC	Benign	NS	NS	Frozen	ND	Somatic	*HRAS*	c.182A>G, p.Gln61Arg	01/2009	10/2009	Alive, disease free			
60	145	F	51	PCC	Benign	sympt. (local mass)	A	Frozen	ND	Somatic	*NF1*	c.6855C>A, p.Tyr2285*	11/2010	11/2010	Alive, post-surgery			
61	150	M	26	PCC	Benign	sympt. (adrenergic)	A	Frozen	ND	No			10/2011	01/2013	Alive, disease free			
62	177	F	37	PCC	Benign	sympt. (adrenergic)	High, but NS	Frozen	ND	No			01/2002	02/2014	Alive, disease free			
63	243	M	16	A-PGL	Benign	sympt. (adrenergic)	High, but NS	FFPE	ND	Germline	*SDHB*	c.166_170delCCTCA, p.Pro56delTyrfs*5	06/2005	04/2013	Alive, disease free			
64	183	F	48	A-PGL	Benign	inc. (surgery)	NS	Frozen	ND	No			01/2002	02/2006	Alive, disease free			
65	251	M	53	HN-PGL	Benign	NS	NS	No	ND	Germline	*SDHD*	c.334_337delACTG, p.Asp113Metfs*21	01/2001	06/2008	Alive, disease free			
66	205	F	63	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen	ND	No			03/2003	01/2006	Alive, disease free			

67	286	M	61	A-PGL	Benign	inc. (surgery)	NS	Frozen	ND	No			11/2005	02/2006	Alive, disease free			
68	288	M	59	A-PGL	Benign	inc. (surgery)	NS	Frozen	ND	No			01/2003	01/2006	Alive, disease free			
69	305	M	75	A-PGL	Benign	inc. (image)	A	Frozen	ND	No			04/2007	12/2009	Alive, disease free			
70	403	M	56	A-PGL	Benign	inc. (image)	Nad.	Frozen	ND	Somatic	*SDHB*	c.464C>G, p.Pro155Arg	10/2010	07/2013	Alive, disease free			
71	278	M	12	A-PGL	Benign	NS	NS	No	ND	Germline	*SDHB ‡/Δ*	c.166_170delCCTCA, p.Pro56delTyrfs*5	11/2005	11/2005	Alive, post-surgery			
72	464	F	24	A-PGL	Benign	sympt. (adrenergic)	Nad.	Frozen	ND	No			03/2012	08/2013	Alive, disease free			
73	475	F	78	PCC	Benign	inc. (image)	High, but NS	Frozen	ND	Somatic	*HRAS*	c.182A>G, p.Gln61Arg	06/2012	03/2013	Alive, disease free			
74	480	F	51	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen	ND	Somatic	*VHL*	c.389T>G, p.Val130Gly	07/2012	11/2012	Alive, disease free			
75	601	F	65	PCC	Benign	sympt. (adrenergic)	A	Frozen	ND	No			06/2007	09/2008	Alive, unknown			
76	513	M	19	PCC	Benign	inc. (image)	High, but NS	FFPE	Positive	Somatic	*VHL*	c.475A>G, p.Lys159Glu	10/2012	05/2013	Alive, disease free			
77	628	M	52	PCC	Benign	sympt. (adrenergic)	High, but NS	Frozen	ND	No			01/2010	01/2010	Alive, post-surgery			
78	619	M	14	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen	ND	Somatic	*VHL*	c.496G>T, p.Val166Phe	03/2009	03/2009	Alive, post-surgery			
79	751	F	48	PCC	Benign	NS	NS	Frozen	ND	Somatic	*RET*	c.2753T>C, p.Met918Thr	01/2003	11/2008	Alive, disease free			
80	764	F	68	PCC	Benign	inc. (image)	No sec.	Frozen	ND	Somatic	*HRAS ◊*	c.182A>G, p.Gln61Arg	03/2010	05/2013	Alive, disease free			

81	893	F	63	PCC	Benign	sympt. (adrenergic)	A	Frozen (finished)	ND	No			07/2013	09/2013	Alive, disease free			
82	285	M	59	HN-PGL	Benign	NS	NS	Frozen	ND	No			07/2005	07/2005	Alive, post-surgery			
83	407	F	29	HN-PGL	Benign	sympt. (local mass)	NS	Frozen	ND	No			10/2010	03/2012	Alive, disease free			
84	301	M	27	HN-PGL	Malignant	inc. (surgery)	No sec.	No	ND	Germline	*SDHB*	c.166_170delCCTCA, p.Pro56delTyrfs*5	10/2001	11/2012	Alive, disease free	Local lymph node	Sync.	AP study
85	100	F	56	PCC	Benign	sympt. (adrenergic)	High, but NS	Frozen and FFPE	Positive	No			07/2002	02/2014	Alive, disease free			
86	136	F	42	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen and FFPE	Positive	No			06/2009	01/2014	Alive, disease free			
87	307	M	40	HN-PGL	Benign	NS	No sec.	No	ND	Germline	*SDHD Δ*	c.168_169delTT, p.Ser57Trpfs*11	08/2007 diagnosis, surgery unknown	08/2007	Alive, unknown			
88	152	F	54	PCC	Benign	inc. (surgery)	A	Frozen and FFPE	Positive	No			09/2011	07/2013	Alive, disease free			
89	311	F	22	A-PGL	Malignant	sympt. (local mass)	No sec.	No	ND	Germline	*SDHD Δ*	c.210G>T, p.Arg70Ser	02/2006	09/2013	Alive, met.	Local lymph nodes and lung	Sync.	AP study
90	312	F	32	T-PGL	Malignant	sympt. (adrenergic)	Nad.	Frozen	ND	Germline	*SDHB* Δ	c.166_170delCCTCA, p.Pro56delTyrfs*5	10/2007	11/2011	Alive ,met.	Bone	Sync.	AP study
91	165	F	69	HN-PGL	Benign	sympt. (local mass)	NS	Frozen and FFPE	Positive	No			06/2002	06/2002	Alive, post-surgery			
92	327	M	14	A-PGL	Benign	sympt. (adrenergic)	NS	Frozen	ND	Germline	*SDHB*-GD ‡	exon 1 gross deletion	03/2007	07/2008	Alive, disease free			

93	631	M	35	PCC	Benign	inc. (image)	Nad.	Frozen and FFPE	Positive	Somatic	*VHL*	c.260T>C, p.Val87Ala	01/2010	08/2010	Alive, disease free			
94	330	M	40	A-PGL	Benign	sympt. (local mass)	NS	FFPE	ND	Germline	*SDHB Δ*	c.424-3C>G	04/2008	06/2008	Alive, disease free			
95	268	F	53	A-PGL	Benign	sympt. (adrenergic)	High, but NS	Frozen and FFPE	Positive	No			07/2004	09/2006	Alive, disease free			
96	418	M	64	A-PGL	Malignant	inc. (image)	Nad.	Frozen and FFPE	Positive	No			12/2010	03/2014	Alive, met.	Local lymph node	Sync.	AP study
97	538	M	46	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen and FFPE	Positive	No			03/2004	12/2004	Alive, disease free			
98	340	F	38	HN-PGL	Benign	NS	NS	No	ND	Germline	*SDHD*	c.2T>C, p.Met1?	01/2008	11/2008	Alive, unknown			
99	550	M	45	PCC	Benign	sympt. (adrenergic)	A	Frozen and FFPE	Positive	Somatic	*HRAS ◊*	c.182A>G, p.Gln61Arg	04/2004	07/2014	Alive, disease free			
100	553	M	57	PCC	Benign	NS	NS	Frozen and FFPE	Positive	Somatic	*RET*	c.2753T>C, p.Met918Thr	10/2004	01/2005	Alive, disease free			
101	581	M	36	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen and FFPE	Positive	Somatic	*VHL*	c.482G>A, p.Arg161Gln	01/2006	02/2014	Alive, disease free			
102	635	F	30	PCC	Benign	sympt. (adrenergic)	High, but NS	Frozen and FFPE	Positive	Somatic	*VHL*	c.491A>G, p.Gln164Arg	01/2002	01/2005	Alive, disease free			
103	728	F	46	PCC	Benign	NS	NS	Frozen	ND	Somatic	*EPAS1**	c.1599_1604delCCCCAT, p.Ile533_Pro534del	01/2003	10/2010	Alive, disease free			
104	643	F	27	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen and FFPE	Positive	No			02/2011	11/2011	Alive, disease free			
105	727	F	78	A-PGL	Benign	NS	NS	Frozen and FFPE	Positive	Somatic	*EPAS1**	c.1615G>T, p.Asp539Tyr	07/2003	07/2003	Alive, post-surgery			
106	759	F	42	PCC	Benign	sympt. (adrenergic)	Nad.	Frozen and FFPE	Positive	No			12/2012	12/2012	Alive, post-surgery			

107	352	F	46	A-PGL	Malignant	inc. (image)	NS	No	ND	Germline	*SDHB*	c.725G>A, p.Arg242His	No surgery, 06/2008 (only biopsy)	03/2009	Alive, met. (no surgery)	Liver and local lymph node	Sync.	AP study
108	353	M	32	HN-PGL	Malignant	sympt. (local mass)	No sec.	No	ND	Germline	*SDHB*	c.269G>A, p.Arg90Gln	02/2008	04/2008	Alive, disease free	Bone	Sync.	AP study
109	760	M	58	PCC	Benign	NS	A	Frozen and FFPE	Positive	Somatic	*RET*	c.2753T>C, p.Met918Thr	01/2013	01/2013	Alive, post-surgery			
110	364	F	67	HN-PGL	Benign	sympt. (adrenergic)	No sec.	No	ND	Germline	*SDHB*	c.557G>A, p.Cys186Tyr	No surgery, diagnosis 02/2009	09/2009	Alive, stable disease (no surgery)			
111	365	M	25	HN-PGL	Benign	NS	No sec.	No	ND	Germline	*SDHB*	c.166_170delCCTCA, p.Pro56delTyrfs*5	01/2009	10/2009	Alive, disease free			
112	368	M	23	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	Germline	*SDHB*	c.166_170delCCTCA, p.Pro56delTyrfs*5	06/2006	11/2009	Alive, disease free			
113	967	F	38	A-PGL	Benign	sympt. (adrenergic)	Nad.	Frozen and FFPE	Positive	Somatic	*EPAS1*	c.1592C>T, p.Pro531Leu	10/2013	10/2013	Alive, post-surgery			
114	1005	M	65	PCC	Benign	NS	NS	Frozen and FFPE	Positive	No			11/2010	11/2010	Alive, post-surgery			
115	405	F	48	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			10/2009	10/2010	Alive, local recurrence			
116	498	F	27	PCC	Benign	inc. (image)	Nad.	FFPE	Positive	Somatic	*VHL*	c.227T>A, p.Phe76Tyr	06/2012	01/2013	Alive, disease free			
117	501	F	78	PCC	Benign	inc. (image)	Nad.	No	ND	No			07/2014	07/2014	Alive, post-surgery			
118	1015	M	51	PCC	Benign	inc. (image)	Nad.	No	ND	No			12/2010	03/2014	Alive, disease free			

119	1020	F	53	PCC	Benign	inc. (image)	Nad.	No	ND	No			05/2014	06/2014	Alive, post-surgery			
120	1023	M	78	A-PGL	Benign	inc. (image)	Nad.	No	ND	No			02/2014	02/2014	Alive, post-surgery			
121	1024	F	35	PCC	Benign	NS	Nad.	No	ND	No			01/2012	04/2014	Alive, disease free			
122	5	F	56	A-PGL	Benign	inc. (surgery)	NS	No	ND	No			12/2005	11/2006	Alive, disease free			
123	35	F	62	PCC	Benign	sympt. (adrenergic)	High, but NS	No	ND	No			12/1994	01/2006	Alive, disease free			
124	400	F	42	HN-PGL	Benign	NS	NS	No	ND	Germline	*SDHB*-GD	exon 1 gross deletion	No surgery, diagnosis 01/2010	09/2010	Alive, stable disease (no surgery)			
125	56†	F	56	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			11/1998	01/2010	Deceased (liposarcoma, but PPGL free)			
126	64	F	34	PCC	Benign	sympt. (local mass)	A	No	ND	No			04/1999	03/2014	Alive, disease free			
127	65	F	62	A-PGL	Benign	inc. (image)	High, but NS	No	ND	No			07/1999	01/2013	Alive, disease free			
128	77	F	37	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			12/1992	02/2014	Alive, disease free			
129	79	F	42	PCC	Benign	NS	NS	No	ND	No			01/2000	01/2000	Alive, post-surgery			
130	80	M	40	PCC	Benign	NS	NS	No	ND	No			01/2000	01/2000	Alive, post-surgery			

131	82	M	23	PCC	Benign	inc. (image)	A	No	ND	No			01/1998	01/2006	Alive, disease free			
132	413	F	44	HN-PGL	Benign	NS	NS	No	ND	Germline	*SDHB*	c.544_550delGGGCTCT, p.Gly182Thrfs*36	12/2010 diagnosis, surgery unknown	12/2010	Alive, unknown			
133	92	F	42	PCC	Benign	NS	NS	No	ND	No			01/2002	01/2002	Alive, post-surgery			
134	97	F	48	PCC	Benign	inc. (surgery)	NS	No	ND	No			01/1997	01/2006	Alive, disease free			
135	98	M	31	PCC	Benign	NS	NS	No	ND	No			11/2002	11/2002	Alive, post-surgery			
136	99	F	59	PCC	Benign	NS	Nad.	No	ND	No			01/1987	03/2012	Alive, disease free			
137	107	F	NS	PCC	Benign	NS	NS	No	ND	No			01/2004	01/2004	Alive, post-surgery			
138	108	F	63	PCC	Benign	NS	NS	No	ND	No			01/2004	01/2004	Alive, post-surgery			
139	121	F	39	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			11/2004	09/2005	Alive, disease free			
140	424	F	26	A-PGL	Benign	sympt. (adrenergic)	Nad.	FFPE	Negative and negative SDHA-IHC	Germline	*SDHA*	c.1754G>A, p.Arg585Gln	12/2007	03/2012	Alive, disease free			
141	425	F	53	HN-PGL	Benign	inc. (image)	Nad.	No	ND	No			No surgery, 12/2009 (only biopsy)	03/2012	Alive, increasing disease (no surgery)			
142	123	F	34	PCC	Benign	NS	NS	No	ND	No			01/2007	06/2007	Alive, disease free			
143	129	F	55	PCC	Benign	NS	NS	No	ND	No			01/2007	02/2008	Alive, disease free			

144	430	M	37	A-PGL	Benign	NS	NS	No	ND	Germline	*SDHB*-GD	exon 1 gross deletion	01/2010	04/2011	Alive, disease free			
145	131	F	21	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			01/1994	04/2009	Alive, disease free			
146	134	F	59	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			11/1994	01/2010	Alive, disease free			
147	433	M	60	T-PGL	Malignant	inc. (image)	Nad.	FFPE	ND	Germline	*SDHB*	c.287-3C>G	No surgery, 12/2010 (only biopsy)	01/2011	Alive, met. (no surgery)	Bone and local lymph node	Sync.	AP study
148	135	M	36	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			11/2007	07/2010	Alive, disease free			
149	139	F	28	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			02/2002	03/2010	Alive, disease free			
150	441	M	28	HN-PGL	Benign	NS	NS	No	ND	Germline	*SDHD*	c.191_192delTC, p.Leu64Profs*4	No surgery, diagnosis 01/2010	06/2011	Alive, stable disease (no surgery)			
151	442	M	20	A-PGL	Benign	sympt. (adrenergic)	Nad.	Frozen	ND	Germline	*SDHB*	c.423+1G>A	01/2011	06/2011	Alive, local residual disease			
152	444	F	40	HN-PGL	Benign	NS	A	FFPE	Negative	Germline	*SDHAF2*	c.362G>A, p.Trp121*	02/2007	02/2012	Alive, disease free			
153	146	M	45	PCC	Benign	NS	NS	No	ND	No			01/2009	10/2010	Alive, disease free			
154	147	M	67	PCC	Benign	sympt. (local mass)	NS	No	ND	No			01/2008	03/2011	Alive, disease free			
155	449	F	15	A-PGL	Benign	sympt. (adrenergic)	Nad.	Frozen	ND	Germline	*SDHA*	c.457-1A>G	11/2011	12/2011	Alive, post-surgery			
156	148	F	64	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			02/2011	03/2011	Alive, post-surgery			

157	149	M	36	PCC	Benign	inc. (image)	Nad.	No	ND	No			02/2011	10/2014	Alive, disease free			
158	158	F	36	HN-PGL	Benign	inc. (surgery)	No sec.	No	ND	No			11/2000	11/2006	Alive, disease free			
159	168	F	43	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/1998	04/2006	Alive, disease free			
160	179	F	69	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			12/2002	12/2002	Alive, post-surgery			
161	180	F	51	PCC	Benign	sympt. (adrenergic)	High, but NS	No	ND	No			02/2002	02/2006	Alive, disease free			
162	189	M	72	A-PGL	Benign	inc. (image)	A	No	ND	No			02/2001	12/2003	Alive, disease free			
163	190	M	40	PCC	Benign	inc. (image)	Nad.	No	ND	No			03/2002	01/2013	Alive, disease free			
164	191	F	50	T-PGL	Benign	NS	NS	No	ND	No			01/1997	01/2003	Alive, disease free			
165	192	F	27	PCC	Benign	NS	NS	No	ND	No			06/1988	01/2002	Alive, disease free			
166	203	M	48	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			03/1997	09/2003	Alive, disease free			
167	206	F	45	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			04/2003	05/2013	Alive, disease free			
168	238	F	68	PCC	Benign	inc. (image)	Nad.	No	ND	No			04/2001	03/2014	Alive, disease free			
169	250	F	21	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	No			10/2004	10/2004	Alive, post-surgery			
170	266	F	37	HN-PGL	Benign	NS	Nad.	No	ND	No			01/1999 diagnosis, surgery unknown	06/2005	Alive, unknown			

171	267	F	28	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			09/2004	09/2004	Alive, post-surgery			
172	270	F	47	PCC	Benign	inc. (image)	A	No	ND	No			07/2005	07/2005	Alive, post-surgery			
173	271	F	26	A-PGL	Benign	NS	High, but NS	No	ND	No			07/2005	07/2005	Alive, post-surgery			
174	282	M	32	PCC	Benign	NS	NS	No	ND	No			11/2004	09/2005	Alive, disease free			
175	479	F	33	HN-PGL	Benign	NS	No sec.	No	ND	Germline	*SDHB*	c.419T>A, p.Val140Asp	01/2012	07/2012	Alive, disease free			
176	284	F	72	T-PGL	Benign	inc. (image)	No sec.	No	ND	No			No surgery, 09/2005 (only biopsy)	03/2014	Alive, stable disease (no surgery)			
177	289	F	65	HN-PGL	Benign	NS	NS	No	ND	No			01/1997	01/1997	Alive, post-surgery			
178	483	F	50	T-PGL	Malignant	inc. (surgery)	No sec.	No	ND	Germline	*SDHC*	c.43C>T, p.Arg15*	05/2012	05/2013	Alive, disease free	Bone	Sync.	AP study
179	290	F	46	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2005	06/2006	Alive, disease free			
180	485	F	14	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	Germline	*SDHB*-GD ‡	exon 1 gross deletion	12/2009	02/2012	Alive, disease free			
181	487	M	22	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	Germline	*SDHB*-GD	exon 1 gross deletion	05/2012	09/2012	Alive, disease free			
182	292	F	60	HN-PGL	Benign	NS	No sec.	No	ND	No			No surgery, diagnosis 01/1985	06/2006	Alive, stable disease (no surgery)			
183	293†	F	72	T-PGL	Benign	NS	NS	No	ND	No			01/2002	03/2014	Deceased (unknown cause,			

															but PPGL free)			
184	298	F	37	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			01/2007	01/2007	Alive, post-surgery			
185	306	F	36	HN-PGL	Benign	inc. (surgery)	NS	No	ND	No			07/2006	07/2006	Alive, post-surgery			
186	497	F	23	T-PGL	Benign	NS	No sec.	No	ND	Germline	*SDHB Δ*	c.643-2A>C	01/2011	11/2012	Alive, disease free			
187	310†	M	57	PCC	Benign	inc. (surgery)	NS	No	ND	No			02/2007	08/2012	Deceased (adverse effects to chemotherapy)			
188	500	M	46	HN-PGL	Benign	NS	NS	No	ND	Germline	*SDHB*	c.127G>C, p.Ala43Pro	09/2012 diagnosis, surgery unknown	09/2012	Alive, unknown			
189	314	F	29	HN-PGL	Benign	NS	NS	No	ND	No			01/2007	12/2007	Alive, disease free			
190	329	F	16	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2007	11/2008	Alive, disease free			
191	336	M	31	T-PGL	Benign	sympt. (adrenergic)	High, but NS	No	ND	No			01/2006	01/2006	Alive, post-surgery			
192	338	M	50	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2007	02/2014	Alive, disease free			
193	342	F	43	HN-PGL	Benign	sympt. (local mass)	A	No	ND	No			11/2008	07/2013	Alive, disease free			
194	345	F	59	PCC	Benign	NS	NS	No	ND	No			11/2006	12/2008	Alive, disease free			
195	510†	M	71	A-PGL	Malignant	sympt. (local mass)	Nad.	FFPE	Negative and	Germline	*SDHA*	c.457-1A>G	04/2013	12/2013	Deceased (radioth	Bone	Sync.	AP study

									negative IHC SDHA						erapy bone met.)			
196	346	F	NS	PCC	Benign	NS	NS	No	ND	No			12/2008	12/2008	Alive, post-surgery			
197	347	M	59	PCC	Benign	NS	NS	No	ND	No			01/2008	11/2008	Alive, disease free			
198	348	F	35	PCC	Benign	NS	NS	No	ND	No			01/2003	12/2008	Alive, disease free			
199	349	F	NS	PCC	Benign	NS	NS	No	ND	No			12/2008	12/2008	Alive, post-surgery			
200	350	M	NS	PCC	Benign	NS	NS	No	ND	No			12/2008	09/2013	Alive, disease free			
201	359	F	62	T-PGL	Benign	NS	No sec.	No	ND	No			01/2009	05/2009	Alive, post-surgery			
202	370	F	57	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			01/2010	05/2014	Alive, disease free			
203	373	F	54	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			11/2009	03/2010	Alive, disease free			
204	541	F	65	PCC	Benign	inc. (image)	Nad.	No	ND	Germline	*SDHB Δ*	c.725G>A, p.Arg242His	08/2004	10/2004	Alive, disease free			
205	376	F	77	HN-PGL	Benign	NS	NS	No	ND	No			12/2009	03/2010	Alive, disease free			
206	385	M	43	HN-PGL	Benign	NS	No sec.	No	ND	No			06/2010	05/2014	Alive, disease free			
207	386	F	38	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			01/1997	05/2014	Alive, disease free			
208	387	M	69	HN-PGL	Benign	NS	NS	No	ND	No			01/2010 diagnosis, surgery unknown	06/2010	Alive, unknown			

209	389	M	15	PCC	Benign	NS	NS	No	ND	No			01/2007	06/2010	Alive, disease free			
210	393	M	32	PCC	Benign	NS	NS	No	ND	No			01/2009	07/2010	Alive, disease free			
211	394†	M	47	PCC	Malignant	sympt. (adrenergic)	No sec.	No	ND	No			02/2010	07/2010	Deceased (met.)	Liver and local lymph node	Sync.	AP study
212	401	F	78	HN-PGL	Benign	NS	NS	No	ND	No			08/2010 diagnosis, surgery unknown	08/2010	Alive, unknown			
213	402	F	60	HN-PGL	Benign	NS	High, but NS	No	ND	No			10/2010 diagnosis, surgery unknown	10/2010	Alive, unknown			
214	410	F	45	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			11/2010	05/2014	Alive, disease free			
215	412	F	68	HN-PGL	Benign	NS	NS	No	ND	No			01/2007 diagnosis, surgery unknown	12/2010	Alive, unknown			
216	417	F	70	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			01/2011 (palliative surgery)	05/2014	Alive, stable disease (palliative surgery)			
217	420	F	53	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			03/2011	05/2014	Alive, disease free			
218	421	F	41	PCC	Malignant	sympt. (local mass)	A	No	ND	No			01/1993	04/2011	Alive,met.	Bone and lung	Metac., 120 months after first surgery	Image (CT) and BC study

219	422	F	25	A-PGL	Benign	sympt. (local mass)	Nad.	No	ND	No			01/2010	02/2011	Alive, disease free			
220	578	F	26	PCC	Benign	sympt. (adrenergic)	A	FFPE	Not available	No			06/2006	06/2006	Alive, post-surgery			
221	423	F	26	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	No			05/2006	03/2011	Alive, disease free			
222	427	F	41	PCC	Benign	inc. (image)	Nad.	No	ND	No			02/2011	03/2011	Alive, disease free			
223	428	M	38	PCC	Benign	NS	A	No	ND	No			10/2010	08/2011	Alive, disease free			
224	431	M	38	A-PGL	Benign	NS	NS	No	ND	No			01/2011	01/2011	Alive, post-surgery			
225	432	F	38	A-PGL	Benign	NS	NS	No	ND	No			11/2010	05/2011	Alive, disease free			
226	438	F	46	T-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	No			06/2001	06/2013	Alive, disease free			
227	439	F	64	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			06/2011	05/2014	Alive, disease free			
228	445	F	58	A-PGL	Benign	inc. (surgery)	NS	No	ND	No			07/2011	07/2013	Alive, disease free			
229	448	M	42	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			10/2011	01/2012	Alive, disease free			
230	451	M	54	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			12/2011	05/2014	Alive, disease free			
231	457	F	43	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			02/2012	02/2012	Alive, post-surgery			
232	458	F	NS	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			02/2012	02/2012	Alive, post-surgery			

233	459	F	46	HN-PGL	Benign	NS	NS	No	ND	No			11/2011	02/2012	Alive, disease free			
234	463	M	45	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2007	03/2012	Alive, disease free			
235	466	F	50	PCC	Benign	NS	NS	No	ND	No			01/2011	01/2012	Alive, disease free			
236	469	F	52	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			05/2012	05/2014	Alive, disease free			
237	472	F	63	HN-PGL	Benign	NS	NS	No	ND	No			05/2012 diagnosis, surgery unkown	05/2012	Alive, unknown			
238	473	M	54	HN-PGL	Benign	NS	NS	No	ND	No			12/2011	03/2012	Alive, disease free			
239	474	M	49	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			06/2012	05/2014	Alive, disease free			
240	476	M	70	PCC	Benign	sympt. (adrenergic)	High, but NS	No	ND	No			No surgery, diagnosis 01/2011	05/2012	Alive, stable disease (no surgery)			
241	489	M	59	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			09/2012	05/2014	Alive, disease free			
242	491	M	60	A-PGL	Benign	sympt. (local mass)	Nad.	No	ND	No			03/2011	03/2014	Alive, disease free			
243	495	F	55	HN-PGL	Benign	sympt. (local mass)	NS	No	ND	No			11/2012	12/2012	Alive, post-surgery			
244	615	M	70	PCC	Benign	inc. (image)	A	No	ND	Germline	*RET*	c.2410G>T, p.Val804Leu	05/2008	03/2013	Alive, disease free			
245	616	F	18	PCC	Benign	NS	Nad.	No	ND	Germline	*VHL ǂ*	c.482G>A, p.Arg161Gln	01/1997	10/2008	Alive, disease free			

246	502	F	50	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			01/2013	05/2014	Alive, disease free			
247	506	M	64	PCC	Benign	inc. (image)	A	No	ND	No			08/2012	08/2012	Alive, post-surgery			
248	509	F	39	HN-PGL	Benign	sympt. (local mass)	No sec.	No	ND	No			07/2012	03/2013	Alive, disease free			
249	511	M	63	HN-PGL	Benign	inc. (image)	Nad.	No	ND	No			03/2013	03/2013	Alive, post-surgery			
250	515	F	65	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			04/2013	06/2013	Alive, disease free			
251	537†	M	62	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			07/2003	09/2011	Deceased (pancreatic adenocarcinoma)			
252	539	F	43	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			01/2004	01/2004	Alive, post-surgery			
253	540	M	37	PCC	Benign	NS	NS	No	ND	No			07/2003	04/2004	Alive, disease free			
254	552	F	80	PCC	Benign	NS	NS	No	ND	No			01/2001	01/2001	Alive, post-surgery			
255	626	F	26	PCC	Benign	sympt. (adrenergic)	A	FFPE	ND	Germline	*TMEM127*	c.115_118delCTGT, p.Ile41Argfs*39	01/2008	07/2010	Alive, disease free			
256	555	F	71	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			03/2005	12/2005	Alive, disease free			
257	557	F	31	PCC	Benign	NS	NS	No	ND	No			03/1998	04/2005	Alive, disease free			
258	558	F	35	PCC	Benign	NS	NS	No	ND	No			01/1997	01/2005	Alive, disease free			

259	633	M	35	PCC	Benign	sympt. (adrenergic)	Nad.	FFPE	Positive	Germline	*TMEM127*	c.221A>C, p.Tyr74Ser	11/2009	12/2012	Alive, disease free			
260	559	M	48	PCC	Benign	sympt. (adrenergic)	High, but NS	No	ND	No			02/2005	03/2005	Alive, post-surgery			
261	560	F	74	PCC	Benign	inc. (image)	Nad.	No	ND	No			07/2005	06/2013	Alive, disease free			
262	562	F	43	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			12/2002	10/2005	Alive, disease free			
263	563	F	33	PCC	Benign	NS	NS	No	ND	No			09/1993	12/2005	Alive, disease free			
264	569	M	36	PCC	Benign	inc. (image)	Nad.	No	ND	No			12/2005	05/2011	Alive, disease free			
265	640	M	26	PCC	Malignant	NS	Nad.	No	ND	Germline	*SDHB*-GD	exon 1 gross deletion	03/2010	04/2010	Alive, post-surgery	Bone	Sync.	AP study
266	570	M	40	PCC	Malignant	NS	Nad.	No	ND	No			01/1972	01/2012	Alive, met.	Bone	Metac., 168 months after first surgery	Image (MIBG) and BC study
267	571	F	47	PCC	Benign	inc. (image)	High, but NS	No	ND	No			11/1999	02/2006	Alive, disease free			
268	575	M	56	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			02/2006	01/2013	Alive, disease free			
269	577	F	66	PCC	Benign	NS	NS	No	ND	No			01/2004	01/2006	Alive, disease free			
270	579	M	44	PCC	Benign	NS	NS	No	ND	No			11/2006	12/2006	Alive, disease free			
271	580	M	47	A-PGL	Benign	sympt. (adrenergic)	Nad.	No	ND	No			03/2007	05/2007	Alive, disease free			

272	588	M	33	PCC	Benign	NS	NS	No	ND	No			01/2006	04/2007	Alive, disease free			
273	590	M	41	PCC	Benign	sympt. (adrenergic)	High, but NS	No	ND	No			01/2006	11/2007	Alive, disease free			
274	593	F	46	PCC	Benign	inc. (image)	NS	No	ND	No			11/2006	05/2007	Alive, disease free			
275	595	F	51	PCC	Benign	NS	NS	No	ND	No			01/2007	06/2007	Alive, disease free			
276	596	F	62	PCC	Benign	NS	NS	No	ND	No			01/2007	07/2007	Alive, disease free			
277	598	F	44	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2007	07/2007	Alive, disease free			
278	603	M	61	PCC	Benign	NS	NS	No	ND	No			01/2007	11/2007	Alive, disease free			
279	604	M	24	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			04/2007	01/2010	Alive, disease free			
280	671†	M	46	PCC	Malignant	NS	Nad.	FFPE	ND	Germline	*SDHB*	c.112C>T, p.Arg38*	01/2000	01/2000	Deceased (unknown)	Liver and lung	Metac., 28 months after first surgery	Image (MIBG) and BC study
281	715	M	29	HN-PGL	Malignant	NS	NS	FFPE	ND	Germline	*SDHD*	c.334_337delACTG, p.Asp113Metfs*21	03/2001	07/2011	Alive, disease free	Bone	Sync.	AP study
282	605	M	55	PCC	Benign	sympt. (adrenergic)	High, but NS	No	ND	No			03/2007	05/2008	Alive, disease free			
283	607	M	44	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			02/2006	07/2011	Alive, disease free			
284	608	F	54	PCC	Benign	NS	NS	No	ND	No			01/2008	01/2008	Alive, post-surgery			

285	609	F	39	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			06/2007	02/2014	Alive, disease free			
286	610†	M	54	PCC	Benign	inc. (image)	NS	No	ND	No			05/2008	08/2008	Deceased (recurrence of the gallbladder adenoca with liver met)			
287	757	M	16	T-PGL	Malignant	sympt. (local mass)	No sec.	No	ND	Germline	*SDHB*-GD	exon 1 gross deletion	No surgery, 05/2013 (only biopsy)	06/2013	Alive, met. (no surgery)	Bone	Sync.	AP study
288	617	F	41	PCC	Benign	inc. (image)	Nad.	No	ND	No			01/2008	01/2008	Alive, post-surgery			
289	618	M	48	PCC	Benign	inc. (image)	Nad.	No	ND	No			09/2008	02/2012	Alive, disease free			
290	620	M	44	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			03/2009	06/2009	Alive, disease free			
291	622	M	43	PCC	Benign	NS	NS	No	ND	No			01/2009	01/2009	Alive, post-surgery			
292	623	M	21	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			07/2009	08/2009	Alive, post-surgery			
293	624	F	19	PCC	Benign	NS	NS	No	ND	No			01/1997	01/2009	Alive, disease free			
294	815	M	13	PCC	Benign	sympt. (adrenergic)	NS	Frozen and FFPE	ND	Germline	*SDHB* ǂ	c.540G>C, p.Leu180Leu	01/1985	05/2005	Alive, disease free			
295	625	M	47	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2010	03/2011	Alive, disease free			
296	638	F	32	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2002	08/2010	Alive, disease free			

297	639	M	44	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			11/2010	10/2012	Alive, disease free			
298	642	F	62	PCC	Benign	NS	Nad.	No	ND	No			01/2009	04/2011	Alive, disease free			
299	644	M	40	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			01/2011	01/2011	Alive, post-surgery			
300	645	M	35	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			07/2010	10/2012	Alive, disease free			
301	650	F	74	PCC	Benign	NS	NS	No	ND	No			01/2011	07/2011	Alive, disease free			
302	894	M	32	PCC	Benign	sympt. (adrenergic)	A	Frozen	ND	Germline	*RET*	c.1998G>C, p.Lys666Asn	07/2013	09/2013	Alive, disease free (only 2 months post-surgery)			
303	651	F	47	PCC	Benign	sympt. (adrenergic)	NS	No	ND	No			04/2008	12/2012	Alive, disease free			
304	652	M	NS	PCC	Benign	NS	NS	No	ND	No			01/2012	01/2012	Alive, post-surgery			
305	965	M	42	PCC	Malignant	inc. (image)	High, but NS	FFPE	Positive	Somatic	*RET*	c.2753T>C, p.Met918Thr	07/2012	08/2013	Alive, disease free	Local lymph node	Sync.	AP study
306	755	M	42	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			01/2012	04/2013	Alive, disease free			
307	756	F	34	A-PGL	Benign	sympt. (adrenergic)	A	No	ND	No			06/2013	06/2013	Alive, post-surgery			
308	803	F	NS	PCC	Benign	NS	NS	No	ND	No			04/1999	04/1999	Alive, post-surgery			
309	804	F	42	PCC	Benign	NS	NS	No	ND	No			01/2000	01/2000	Alive, post-surgery			

310	806†	M	65	A-PGL	Benign	NS	NS	No	ND	No			01/2001	03/2001	Deceased (met. medullary thyroid carcinoma)			
311	881	M	59	PCC	Benign	inc. (image)	A	No	ND	No			06/2012	01/2013	Alive, disease free			
312	887	F	44	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			02/2013	02/2013	Alive, post-surgery			
313	888	F	42	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			02/2012	04/2013	Alive, disease free			
314	959	M	64	A-PGL	Benign	NS	A	No	ND	No			07/2012	11/2012	Alive, disease free			
315	960	M	68	HN-PGL	Benign	NS	NS	No	ND	No			01/2001	06/2013	Alive, disease free			
316	966	F	62	NS PGL	Malignant	NS	NS	No	ND	No			06/2013	09/2013	Alive, disease free (only 3 months post-surgery)	Not specified	Sync.	AP study
317	968	F	67	PCC	Benign	inc. (image)	No sec.	No	ND	No			02/2012	10/2013	Alive, disease free			
318	969	M	48	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			11/2007	12/2013	Alive, disease free			
319	1001	M	39	PCC	Malignant	sympt. (adrenergic)	Nad.	No	ND	No			01/1993	05/2014	Alive, met.	Bone and liver	Metac., 240 months after first surgery	AP study

320	1002	F	31	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			12/2013	02/2014	Alive, disease free			
321	1003	F	43	PCC	Benign	inc. (image)	A	No	ND	No			01/2013	05/2014	Alive, disease free			
322	1006	F	NS	PCC	Benign	NS	NS	No	ND	No			02/2014	02/2014	Alive, post-surgery			
323	1012	M	45	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			11/2012	01/2014	Alive, disease free			
324	1013	M	44	PCC	Benign	NS	NS	No	ND	No			08/2011	07/2014	Alive, disease free			
325	1014	F	52	PCC	Benign	sympt. (adrenergic)	A	No	ND	No			01/2011	07/2014	Alive, disease free			
326	1017	F	23	PCC	Benign	sympt. (adrenergic)	Nad.	No	ND	No			01/2014 diagnosis, surgery unknown	01/2014	Alive, unknown			
327	1019	F	43	PCC	Benign	sympt. (adrenergic)	Dopa.	No	ND	No			10/2013	02/2014	Alive, disease free			
328	1022	F	48	PCC	Benign	inc. (image)	NS	No	ND	No			01/2012	04/2014	Alive, disease free			
329	1025	M	74	HN-PGL	Malignant	sympt. (local mass)	NS	FFPE	Positive	Somatic	*VHL*	c.197_211delinsCTCGTG, p.Val66_Pro71delinsAlaArgAla	01/2014	06/2014	Alive, met.	Bone	Sync.	AP study

Supplementary table S2. Variant interpretation.

Gene	Variant	ID patient	Coding effect	Pathogenicity	Public Databases: - ExAC - COSMIC	Methodology to assess mutations as pathogenic: - Pubmed - In silico analysis: SIFT, Mutation Taster and Polyphen2
EPAS1	c.1592C>T, p.Pro531Leu	335 (S), 657 (S) and 967 (S)	Missense	Mutation	Not reported Not reported	- Previously reported in a case with multiple PGL and erythrocytosis [1]. - SIFT: deleterious (score 0.02). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
EPAS1	c.1606C>A, p.Asp536Tyr	344 (S)	Missense	Mutation	Not reported Not reported	- Previously reported: Hidroxilation point described [1]. - SIFT: deleterious (score 0.02). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
EPAS1	c.1599_1604delCCCCAT, p.Ile533_Pro534del	728 (S)	In-frame	Mutation	Not reported Not reported	- Previously reported: Hidroxilation point described [1].
EPAS1	c.1615G>T, p.Asp539Tyr	727 (S)	Missense	Mutation	Not reported Not reported	- Previously reported: Hidroxilation point described [1]. - SIFT: deleterious (score 0.02). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
HRAS	c.181C>A, p.Gln61Lys	167 (S), 379 (S), 587 (S)	Missense	Mutation	Not reported COSM496 and COSM123649.	- Reported 2 times [2, 3]. - SIFT: deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Benign with a score of 0.012
HRAS	c.182A>G, p.Gln61Arg	118 (S), 133 (S), 460 (S), 475 (S), 636 (S), 647 (S), 659 (S) 550 (S), 658 (S), 764 (S)	Missense	Mutation	Not reported COSM244958 and COSM499	- Reported 2 times [2, 3]. - SIFT: deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Benign with a score of 0.008
HRAS	c.37G>C, p.Gly13Arg	62 (S), 396 (S)	Missense	Mutation	Not reported COSM486 and COSM99938	- Reported 2 times [2, 3]. - SIFT: deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 0.997
NF1	c.6855C>A, p.Tyr2285*	145 (S)	Nonsense	Mutation	0.000008251/0 hom COSM33676 and COSM705652.	
RET	c.1900T>C,	103 (S)	Missense	Mutation	0.000008274/0 hom	- Described in MEN2 syndrome. First reported 1993 [4, 5].

	p.Cys634Arg				COSM 966	- SIFT: deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
RET	c.1998G>C, p.Lys666Asn	894 (G)	Missense	Mutation	0.000008242/0 hom Not reported.	- Described mutations in the same amino acid residue in MEN2 syndrome [5]. - Functional studies have demonstrated that p.K666N mutation is associated with a high level of RET and ERK phosphorylation and a high transforming potential [6]. - It has been described in medullary thyroid carcinoma patients [7]. - SIFT: deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 0.999
RET	c.2410G>T, p.Val804Leu	615 (G)	Missense	Mutation	0.00001569/ 0 hom Not reported	- Described in MEN2 syndrome [5]. First described in 1995 [8]. - SIFT: deleterious (score 0.05). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
RET	c.2753T>C, p.Met918Thr	889 (S), 965 (S), 63 (S), 553 (S), 653 (S), 751 (S), 760 (S)	Missense	Mutation	Not reported COSM965	- Described in MEN2 syndrome [5]. First described in 1994 [9]. - SIFT: deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 0.999
SDHA	c.1754G>A, p.Arg585Gln	424 (G)	Missense	Mutation	0.000008282/0 hom COSM1067147	- Negative SDHB- and SDHA-IHC - LOVD: not reported - SIFT: deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
SDHA	c.457-1G>A, p?	510 (G), 449 (G)	Splice acceptor variant	Mutation	Not reported Not reported	- Negative SDHB- and SDHA-IHC (ID 510) - LOVD: not reported.
SDHAF2	c.362G>A, p.Trp121*	444 (G)	Nonsense	Mutation	Not reported Not reported	- Negative SDHB-IHC - LOVD: not reported.
SDHB	c.166_170delCCTCA, p.Pro56delTyrfs*5	243 (G), 365 (G), 368 (G), 301 (G), 312 (G), 278 (G)	Frameshift	Mutation	Not reported Not reported	- LOVD: Reported 9 times: First time at 2004 [10].
SDHB	c.112C>T, p.Arg38*	671 (G)	Nonsense	Mutation	Not reported Not reported	- LOVD: not described.
SDHB	c.127G>C,	500 (G)	Missense	Mutation	Not reported	- LOVD: Reported 3 times: First report at 2003 [11].

	p.Ala43Pro				Not reported	- SIFT: Tolerated (score 0.19). - Mutation Taster: Disease causing (p-value 0.999) - Polyphen 2: Benign with a score of 0.356
SDHB	c.269G>A, p.Arg90Gln	353 (G)	Missense	Mutation	0.000008315/0 hom Not reported	- LOVD: Reported 3 times: First report at 2006 [12]. - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
SDHB	c.287-3C>G, p?	433 (G)	Splice site	Mutation	- Not reported	- LOVD: Not reported. - Reported 1 time [13]. - We demonstrated the effect on splicing (data not shown).
SDHB	c.423+1G>A, p?	442 (G)	Splice site	Mutation	- Not reported	- LOVD: Reported 9 times: First report at 2003 [14]
SDHB	c.464C>G, p.Pro155Arg	403 (S)	Missense	Mutation	Not reported Not reported	- LOVD: Not reported. - FFPE tumor not available to perform SDHB-IHC. - The second hit was found using SNP array: loss of 1p (data not shown). - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
SDHB	c.544_550delGGGCTC T, p.Gly182Thrfs*36	413 (G)	Frameshift	Mutation	Not reported Not reported Not ensembl.	- LOVD: Not reported.
SDHB	c.557G>A, p.Cys186Tyr	364 (G)	Missense	Mutation	Not reported Not reported	- LOVD: Reported 4 times. First time at 2007[15]. - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
SDHB	c.725G>A, p.Arg242His	352 (G), 541 (G)	Missense	Mutation	0.00002471/0 hom Not reported	- LOVD: Reported 12 times: First time at 2002 [16]. - SIFT: Deleterious (score 0.01). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
SDHB	c.419T>A, p.Val140Asp	479 (G)	Missense	Mutation (VUS)	Not reported Not reported Not ensembl.	- LOVD: Not reported. - A variant in the same amino acid residue has been described 7 times in LOVD: c.418G>T, p.Val140Phe. - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
SDHB	c.278G>A, p.Cys93Tyr	175 (G)	Missense	Mutation	Not reported. COSM1664073	- LOVD: Reported 1 time. First time at 2009: [17]. - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1). - Polyphen 2: Probably damaging with a score of 1.000

SDHB	c.424-3C>G, p?	330 (G)	Splice site	Mutation	- Not reported	- LOVD: Reported 3 times: First time at 2005 [18].
SDHB	c.643-2A>C, p?	497 (G)	Splice site	Mutation	- Not reported	- LOVD: Reported 1 time [17].
SDHB	c.540G>C, p.Leu180Leu	815 (G)	Synonymous	Mutation	0.000008237/0 hom Not reported	- LOVD: Not reported. - In silico tools (ESE-finder) predicted this variant affected splicing. We demonstrated by sequencing cDNA the lack of mutant allele (data not shown).
SDHB	exon 1 deletion	400 (G), 430 (G), 487 (G), 640 (G), 757 (G), 66 (G), 157 (G), 327 (G), 485 (G).	Deletion	Mutation	- -	- Previously reported in familial paraganglioma syndrome [19].
SDHC	c.43C>T, p.Arg15*	483 (G)	Nonsense	Mutation	Not reported Not reported	- LOVD: Reported 4 times: First time at 2007 [20].
SDHC	c.253-255dupTTT, p.Phe85dup	3 (G)	In-frame	Mutation	Not reported Not reported	- LOVD: Reported 2 times. First time at 2008 [21].
SDHD	c.334_337delACTG, p.Asp113Metfs*21	251 (G), 715 (G)	Frameshift	Mutation	Not reported Not reported	- LOVD: Reported 2 times: First time at 2005 [22].
SDHD	c.191_192delTC, p.Leu64Profs*4	441 (G)	Frameshift	Mutation	Not reported Not reported	- LOVD: Reported 3 times: First time at 2001 [23].
SDHD	c.2T>C, p.Met1?	340 (G)	Missense	Mutation	Not reported Not reported	- LOVD: Not reported. - This mutation affects the first methionine and thus the correct processing of the gene. - Start loss
SDHD	c.168_169delTT, p.Ser57Trpfs*11	307 (G)	Frameshift	Mutation	Not reported Not reported	- LOVD: Reported 2 times. First time at 2005 [17].
SDHD	c.210G>T, p.Arg70Ser	311 (G)	Missense	Mutation	Not reported Not reported	- LOVD. Reported 1 time. First time at 2009 [17]. - LOVD: Mutations affecting the same codon (p.Arg70Met; p.Arg70Gly) have been described. Changes affecting this codon destroy hemo interaction and affect the function of the protein. - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
SDHD	c.112C>T, p.Arg38*	1004 (S)	Non-sense	Mutation	Not reported Not reported	- LOVD: Reported 8 times. First time at 2000 [24].

TMEM127	c.115_118delCTGT, p.Ile41Argfs*39	626 (G)	Frameshift	Mutation	Not reported Not reported	
TMEM127	c.221A>C, p.Tyr74Ser	633 (G)	Missense	Mutation	Not reported Not reported	- We found LOH involving wild-type allele in the corresponding tumor DNA. - SIFT: Deleterious (score 0.03). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Benign with a score of 0.058
VHL	c.191G>C, p.Arg64Pro	465 (S)	Missense	Mutation	Not reported Not reported	- The UMD-VHL mutations: Request ID: 190515141147-33 - SIFT: Tolerated (score 0.13). - Mutation Taster: Disease causing (p-value0.999) - Polyphen 2: Probably damaging with a score of 1.000
VHL	c.197_211delinsCTCGT p.Val66_Pro71delinsAlaArgAla	1025 (S)	In-frame	Mutation	Not reported Not reported	- UMD-VHL not reported.
VHL	c.227T>A, p.Phe76Tyr	498 (S)	Missense	Mutation	Not reported COSM14321	- UMD-VHL not reported. - SIFT: Deleterious (score: 0) - Mutation Taster: Disease causing (p-value: 0.974) - Polyphen 2: Probably damaging with a score of 0.935
VHL	c.250G>C, p.Val84Leu	242 (S)	Missense	Mutation	Not reported COSM236660	- The UMD-VHL mutations: Request ID: 190515142416-21 - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value0.549) - Polyphen 2: Benign with a score of 0.017
VHL	c.260T>C, p.Val87Ala	631 (S), 649 (S)	Missense	Mutation	Not reported Not reported	- UMD-VHL not reported. - SIFT: Deleterious (score 0.04). - Mutation Taster: Polymorphism (p-value0.996) - Polyphen 2: Possibly damaging with a score of 0.573
VHL	c.389T>G, p.Val130Gly	480 (S)	Missense	Mutation	Not reported COSM100047	- UMD-VHL not reported. - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 1.000
VHL	c.475A>G, p.Lys159Glu	513 (S)	Missense	Mutation	Not reported COSM144975	- The UMD-VHL mutations: Request ID: 190515142532-25 - SIFT: Deleterious (score 0.03). - Mutation Taster: Disease causing (p-value 0.996) - Polyphen 2: Probably damaging with a score of 0.999
VHL	c.482G>A, p.Arg161Gln	616 (G), 581 (S)	Missense	Mutation	Not reported COSM18097	- The UMD-VHL mutations: Request ID: 190515142607-149 - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 0.999) - Polyphen 2: Probably damaging with a score of 1.000
VHL	c.491A>G, p.Gln164Arg	635 (S)	Missense	Mutation	Not reported COSM14283	- The UMD-VHL mutations: Request ID: 190515142653-45 - SIFT: Tolerated (score 0.13).

						- Mutation Taster: Disease causing (p-value 0.999) - Polyphen 2: Probably damaging with a score of 0.998
VHL	c.496G>T, p.Val166Phe	619 (S)	Missense	Mutation	Not reported COSM17982	- The UMD-VHL mutations: Request ID: 190515142746-49 - SIFT: Deleterious (score 0.03). - Mutation Taster: Disease causing (p-value 0.999) - Polyphen 2: Probably damaging with a score of 0.989
MAX	c.425C>T, p.Ser142Leu	578 (G)	Missense	VUS	0.00001647/0 hom COSM4577970	- Probably non-pathogenic. Although it has been reported two times [25, 26], this variant did not show functional effect on MYC regulation and the aminoacid is located outside the basic helix-loop-helix leucine zipper domain of the MAX protein [27] - SIFT: tolerated (score 0.33). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 0.999
SDHC	c.*90T>C, p?	1017 (G)	Intronic	VUS	Not reported Not reported	- LOVD: Not reported.
SDHB	c.455C>T, p.Ser152Phe	425 (G)	Missense	VUS	0.00005767/0 hom Not reported	- LOVD: Not reported. - FFPE tumor not available to perform SDHB-IHC. - SIFT: Deleterious (score 0). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Benign with a score of 0.167
SDHB	c.49A>G, p.Thr17Ala	619 (G)	Missense	VUS	Not reported Not reported Not ensembl	- LOVD: Reported 1 time: Probably no pathogenicity. - SIFT: Tolerated (score 0.59) -Mutation Taster: Polymorphism (p-value 1) - Polyphen 2: Benign with a score of 0.000
SDHAF2	c.451C>G, p.Gln151Glu	405 (G)	Missense	VUS	Not reported Not reported	- LOVD: not reported. - This change affects a highly conserved residue in the phylogenetic tree. According to bioinformatic prediction tools, this version is considered as probably pathogenic PolyPhen-probably damaging, and it can affect splicing according to ESEfinder tool. However, two other tools (AGVGD and SIFT-tolerated) classified as a benign variant. Therefore, until we cannot show the effect of the change it should be considered as a VUS. The patient left the follow-up and it was not possible to obtain a new blood sample to retain RNA extract and analyze the potential effect on splicing or FFPE tumor to analyze SDHB-IHC. - SIFT: Tolerated (score 0.37). - Mutation Taster: Disease causing (p-value 1) - Polyphen 2: Probably damaging with a score of 0.982

Supplementary table S3. Clinical characteristics of the 21 patients with no amplification of the sample analyzed. FFPE: formalin fixed paraffin-embedded; NA: Not amplified per amplicon (< 20 reads); PCC:pheochromocytoma; PGL: paraganglioma; HN: head and neck; A: abdominal; SDHB-IHC: SDHB immunohistochemistry; ND: no data; WT: Wild type; GM: germline mutation.

Number of patients	ID	Excluded	Sample	Amplicon coverage	Previously studied	Number	Tumor	Sex	Age (years)	Behaviour	Predominant secretion	SDHB-IHC	Type of sample
1	56	Yes	Blood	NA	Yes	Multiple	Multiple PGL (HN)	Female	63	Benign	No secretion	ND	WT
2	66	Yes	Frozen	NA	Yes	Single	PCC	Male	66	Benign	Adrenergic	ND	WT
3	158	Yes	Frozen	NA	Yes	Single	PCC	Female	54	Benign	Adrenergic	Positive	WT
4	159	Yes	Frozen	NA	Yes	Single	PCC	Female	42	Benign	Noradrenergic	Positive	WT
5	160	Yes	Frozen	NA	Yes	Single	PCC	Male	65	Benign	Adrenergic	Positive	WT
6	174	Yes	Frozen	NA	Yes	Single	PCC	Female	58	Benign	Unknown	ND	WT
7	178	Yes	Frozen	NA	Yes	Single	PCC	Male	51	Benign	No data	ND	WT
8	197	Yes	Blood	NA	Yes	Single	PCC	Female	37	Benign	Unknown	ND	WT
9	199	Yes	Blood	NA	Yes	Multiple	Bilateral PCC	Male	11	Benign	Noradrenergic	ND	WT
10	246	Yes	Blood	NA	Yes	Single	PCC	Female	62	Benign	No data	ND	WT
11	250	Yes	Blood	NA	Yes	Single	PCC	Male	41	Benign	No data	ND	WT
12	263	Yes	Blood	NA	Yes	Single	PCC	Female	41	Benign	Noradrenergic	ND	WT
13	271	Yes	Blood	NA	Yes	Single	PCC	Female	62	Benign	Noradrenergic	ND	WT
14	306	Yes	Blood	NA	Yes	Single	PCC (composite)	Female	51	Benign	Unknown	ND	WT
15	324	Yes	Frozen	NA	No	Single	PCC	Male	43	Benign	Adrenergic	ND	WT
16	337	Yes	Frozen	NA	No	Single	PCC	Female	75	Benign	Adrenergic	ND	WT
17	341	Yes	Blood	NA	Yes	Single	PCC (hyperplasia)	Female	No data	Benign	No data	ND	WT
18	415	Yes	FFPE	NA	Yes	Single	A-PGL	Female	50	Benign	Noradrenergic	Positive	WT
19	418	Yes	FFPE	NA	No	Multiple	Multiple PGL (HN)	Female	14	Benign	No secretion	Negative	Control GM *SDHB*
20	442	Yes	FFPE	NA	No	Single	A-PGL	Female	33	Malignant	No data	Negative	WT
21	444	Yes	FFPE	NA	No	Single	A-PGL	Female	19	Benign	Noradrenergic	Negative	WT

Supplementary table S4. Characteristics of the TGPs designed.

		Panel I	Panel II
Variants used as positive controls (unique variants)		534 (73)	337 (56)
Pathogenic category	**Mutation**	17 (16)	13
	VUS	3	1
	Polymorphism	514 (54)	323 (42)
Gene	***SDHB***	10	4
	SDHA	28	24
	SDHD	5	3
	SDHAF2	2	2
	SDHC* and *FH	2	1
	MDH2	5	6
	TMEM127	4	1
	HRAS	1	2
	RET	8	6
	MAX	1	1
	MEN1	2	0
	NF1	3	0
	VHL	0	4
	EPAS1	0	1
Type of variant	**Single base substitution**	65	51
	Small deletion	5	2
	Small duplication	1	2
	Insertion	2	0
	Indel	0	1
Low coverage regions (≤ 50 reads)		*SDHA* (E1), *SDHC* (E2), *MDH2* (E1), *FH* (E1), *TMEM127* (E2), NF1 (E1).	
		SDHA (E10), *SDHAF2* (E1), *RET* (E8), *MAX* (E1), *EGLN1* (E1), *KIF1B* (E1, E9), *NF1* (E7, E40), *MEN1* (E2).	*MAX* (E5), *NF1* (E13, E23, E37, E46).

VUS: Variant of Unknown Significance; E: exon; bp: base pairs.

Supplementary table S5. Control variants previously found by Sanger sequencing used in panel I and II. Hom: Number of patients homozygotes described.

PANEL-I

MUTATIONS

MUT.	ID	Type of sample	Final	Gene	Alt Read Depth	Read Depth	Alt Variant Freq	Consequence	cDNA	Protein
1	72	Blood	Control germline mutation SDHA	SDHA	1919	3927	48.87	splice_acceptor	c.457-1G>A	
2	111	Blood	Control germline mutation SDHA	SDHA	1210	2297	52.68	splice_acceptor	c.457-1G>A	
3	78	Blood	Control germline mutation SDHA	SDHA	987	2028	48.67	missense	c.1754G>A	p.Arg585Gln
4	356	Frozen	Control somatic mutation HRAS	HRAS	68	202	33.7	missense	c.37G>C	p.Gly13Arg
5	300	Blood	Control germline mutation SDHB	SDHB	182	434	42.4	missense	c.725G>A	p.Arg242His
6	12	Frozen	Control SDHB (tumor, no blood)	SDHB	501	711	70.5	frameshift	c.591delC	p.Ser198Alafs*22
7	168	Frozen	Control NF1 (tumor, no blood)	NF1	999	1194	83.7	splice_donor	c.1062+2T>C	
8	363	Frozen	Control NF1 (tumor, no blood)	NF1	142	199	71.4	frameshift	c.4239delT	p.Phe1413Leufs*15
9	171	Frozen	Control NF1 (tumor, no blood)	NF1	493	601	82.4	frameshift	c.7798_7799insA	p.Ser2601Ilefs*7
10	367	Frozen	Control RET (tumor, no blood)	RET	106	198	53.5	missense	c.1901G>A	p.Cys634Tyr
11	297	Blood	Control germline mutation SDHAF2	SDHAF2	270	518	52.3	stop_gained	c.362G>A	p.Trp121*
12	303	Blood	Control germline mutation MEN1	MEN1	24	81	30.8	missense	c.124G>A	p.Gly42Ser
13	298	Blood	Control germline mutation MAX	MAX	410	753	54.7	stop_gained	c.97C>T	p.Arg33*
14	373	Blood	Control germline mutation SDHD	SDHD	254	514	49.5	splice_acceptor	c.53-2A>G	
15	299	Blood	Control germline mutation SDHD	SDHD	296	581	50.9	missense	c.242C>T	p.Pro81Leu
16	277	Blood	Control germline mutation SDHC	SDHC	350	838	41.8	stop_gained	c.43C>T	p.Arg15*
17	302	Blood	Control germline mutation FH	FH	788	1604	49.3	missense	c.575C>T	p.Pro192Leu

VARIANTS OF UNKNOWN SIGNIFICANCE (VUS)

VUS	ID	Type of sample	Final	Gene	Alt Read Depth	Read Depth	Alt Variant Freq	Consequence	cDNA	Protein
1	218	Blood	Control VUS germline SDHB, probably non pathogenic	SDHB	1279	2633	48.6	missense	c.455C>T	p.Ser152Phe
2	179	Frozen	Control VUS TMEM127 in tumor, no blood	TMEM127	998	1089	92.2	missense	c.448G>C	p.Ala150Pro
3	251	Blood	Control VUS germline TMEM127, probably non pathogenic	TMEM127	Not detected: Low coverage region (E2)			synonymous	c.267A>G	p.Thr89Thr

POLYMORPHISMS (SNPs)

SNPs	Unique SNPs	dbSNP ID	Gene	HGVSc HGVSp	Allele Freq	Allele Freq Amr	Allele Freq Asn	Allele Freq Af	Allele Freq Eur	Allele Freq ExAC		ID	Sample		Alt Read Depth	Read Depth	Alt Variant Freq
1	1	rs377134185	SDHA	c.-4A>G	0	0	0	0	0	0.4656% 8 hom		62	Blood	Low coverage region (E1-SDHA)	9	15	60
2	2	rs34635677	SDHA	c.113A>T p.Asp38Val	1	1	0	0	3	3.529% 103 hom		255	Blood		1143	2258	50.8
3												109	Blood		467	844	55.3
4	3	rs1139424	SDHA	c.309A>G p.Ala103Ala	17	20	4	42	9	15.29% 2023 hom		227	Blood		520	1068	49.2
5												291	Blood		693	1425	48.7
6												62	Blood		114	225	50.67
7												175	Frozen		438	838	52.6
8	4	rs6555055	SDHA	c.619A>C p.Arg207Arg	22	23	5	56	11	15.68 % 2372 hom		35	Blood		456	927	49.4
9												58	Blood		980	2353	41.9
10												100	Blood		270	618	43.8
11												107	Blood		627	1617	38.9
12												118	Blood		518	1175	44.3
13												291	Blood		600	1210	49.8
14												88	Blood		513	1152	44.6
15												3	Blood		633	1408	45.1
16												122	Blood		192	515	37.4
17												62	Blood		432	1005	43.4
18												175	Frozen		427	1225	34.9
19	5	rs2115272	SDHA	c.684T>C p.Asn228Asn	22	23	5	56	11	15.65 % 2368 hom		249	Blood		959	1950	49.4
20												35	Blood		537	1179	45.7
21												88	Blood		531	1096	48.5
22												100	Blood		489	989	49.5
23												118	Blood		499	1062	47.1
24												291	Blood		927	2018	46.2
25												107	Blood		1066	2208	48.5
26												122	Blood		474	973	48.7

27												62	Blood		561	1059	53.1
28												3	Blood		2003	4040	49.9
29												262	Blood		963	1958	49.6
30												175	Frozen		658	1463	45.2
31												14	Blood		662	656	100
32												31	Blood		707	707	100
33												24	Blood		488	951	52.7
34												62	Blood		819	820	100
35												109	Blood		427	427	100
36												118	Blood		387	1005	38.7
37												122	Blood		509	511	99.6
38												128	Blood		410	410	100
39												129	Blood		467	467	100
40	6	rs2288461	SDHA	c.771-11A>G	86	88	69	99	88	88.10 % 47377 hom		167	Blood		38	38	100
41												224	Blood		527	1077	49.7
42												255	Blood		1538	1557	99.7
43												291	Blood		951	963	99.7
44												292	Blood		1609	1614	99.9
45												297	Blood		596	1146	52.8
46												117	Blood		622	245	39.5
47												107	Blood		1052	1065	99.7
48												100	Blood		693	693	100
49												88	Blood		529	529	100
50												3	Blood		565	575	100
51	7	rs34771391	SDHA	c.822C>T p.Gly274Gly	1	1	0	2	0.13	0.4620% 3 hom		128	Blood		409	410	99.8
52												35	Blood		893	1886	47.3
53												3	Blood		575	577	100
54												24	Blood		488	953	51.3
55	8	rs1126417	SDHA	c.891T>C p.Pro297Pro	63	70	33	73	75	70.78% 31200 hom		100	Blood		623	1307	47.7
56												31	Blood		903	1778	50.8
57												109	Blood		1774	1778	99.9
58												122	Blood		851	855	99.5

59												224	Blood		529	1076	49.3
60												255	Blood		726	1557	46.8
61												291	Blood		958	962	99.9
62												292	Blood		1609	1611	100
63												297	Blood		584	1144	51.2
64												128	Blood		1681	1685	99.9
65												129	Blood		701	1397	50.3
66												167	Blood		1290	1292	99.8
67												107	Blood		492	1065	46.3
68												118	Blood		864	1861	46.5
69												117	Blood		1575	737	46.9
70												88	Blood		1950	1956	99.9
71												14	Blood		659	662	100
72												62	Blood		800	1708	46.8
73												169	Frozen		920	1778	51.8
74	9	rs7710005	SDHA	c.896-20A>G	22	23	5	55	11	15.66% 2369 hom		88	Blood		360	798	45.1
75												291	Blood		326	701	47.2
76												118	Blood		590	1050	56.2
77												122	Blood		337	692	48.8
78												107	Blood		299	616	49.3
79												62	Blood		563	1071	52.6
80	10	rs142849100	SDHA	c.969C>T p.Gly323Gly	0.23	1	0	0	0.4	0.7017% 12 hom		255	Blood		530	1112	48
81	11	rs144252500	SDHA	c.1002G>A p.Ala334Ala	0.09	0	0	0	0.26	0.07907 % 1 hom		169	Frozen		209	412	50.7
82	12	rs34779890	SDHA	c.1413C>T p.Ile471Ile	0.14	0.28	0	0.2	0.13	0.2199% 1 hom		128	Blood		415	422	98.3
83	13	rs1041949	SDHA	c.1038C>G p.Ser346Ser	22	24	5	59	11	15.97 % 2574 hom		3	Blood		135	317	42.6
84												62	Blood		172	338	50.9
85												107	Blood		291	616	47.3
86												100	Blood		188	394	47.7
87												118	Blood		280	537	52.3
88												122	Blood		177	342	51.8

89												291	Blood		325	701	46.4
90												88	Blood		505	505	100
91												35	Blood		236	466	50.6
92												237	Blood		235	472	49.9
93	14	rs35277230	SDHA	c.1170C>T p.Phe390Phe	9	3	0	36	0.13	3.240% 579 hom		100	Blood		561	1185	47.3
94												3	Blood		219	412	53.2
95												62	Blood		603	2358	25.6
96												88	Blood		710	3289	21.6
97												175	Frozen		332	1436	23.3
98												291	Blood		573	1961	29.6
99	15	rs10039029	SDHA	c.1680G>A p.Thr560Thr	21	23	4	53	11	15.27 % 1873 hom		122	Blood		201	397	50.6
100												118	Blood		769	3815	20.2
101												107	Blood		556	2049	27.5
102												3	Blood		456	1518	30.4
103												35	Blood		613	2653	23.1
104												35	Blood		95	278	34.2
105												88	Blood		110	382	28.9
106												100	Blood		75	215	35.2
107												291	Blood		642	1239	52
108	16	rs77210621	SDHA	c.1752A>G p.Ala584Ala	22	23	4	56	11	15.59% 2311 hom		107	Blood		714	1414	50.7
109												118	Blood		104	318	32.7
110												122	Blood		201	397	50.9
111												62	Blood		120	373	32.3
112												3	Blood		493	1015	48.9
113												175	Frozen		593	1154	51.7
114	17	rs150831951	SDHA	c.1305G>T p.Leu435Leu	1	2	0	0.2	3	1.927% 40 hom		128	Blood		960	1044	92.1
115												55	Blood		85	210	40.9
116	18	rs6960	SDHA	c.1886A>T p.Tyr629Phe	0	0	0	0	0	15.15% 500 hom		175	Frozen		359	673	53.7
117												216	Blood		389	848	46.1
118	19	rs372662724	SDHA	c.1909-14_1909-13delCT	0	0	0	0	0	0.000825 5% 0 hom	Positive SDHB-IHC confirms as a SNP	291	Blood		339	1445	23.5

119	20	rs35549341	SDHA	c.1908+15C>T	5	7	0.17	1	11	Not described		211	Blood		329	688	48.1
120												216	Blood		444	848	52.4
121												122	Blood		205	260	79.2
122												297	Blood		51	81	63
123												167	Blood		674	1411	47.8
124												109	Blood		441	829	53.2
125												268	Blood		172	366	47.1
126												177	Frozen		590	1196	49.4
127	21	rs6961	SDHA	c.1932G>A p.Val644Val	34	32	33	58	20	17.16% 2406 hom		35	Blood		1190	1851	64.3
128												50	Blood		1265	2043	62.1
129												3	Blood		1203	2211	54.7
130												31	Blood		875	2223	39.4
131												62	Blood		779	1486	52.6
132												100	Blood		1360	1884	72.2
133												224	Blood		922	2499	36.9
134												255	Blood		563	2362	23.9
135												291	Blood		880	1443	61
136												292	Blood		871	2604	33.5
137												297	Blood		482	1933	25
138												107	Blood		249	524	48
139												118	Blood		1438	2258	63.7
140												122	Blood		983	1523	64.5
141												128	Blood		362	1361	26.7
142												129	Blood		639	1683	38
143												167	Blood		735	2145	34.3
144												109	Blood		724	1971	36.8
145												88	Blood		958	1500	63.9
146												24	Blood		760	2093	36.4
147												14	Blood		679	1900	35.9
148												58	Blood		2173	3208	67.9
149												175	Frozen		384	524	73.3
150	22	rs148627127	SDHA	c.1944_1945delTT p.Leu649Glufs*4	3	3	6	3	1	1.35 % 0 hom		122	Blood		435	1523	28.6
151												297	Blood		483	1935	25

152												100	Blood		511	1886	27.1
153	23	rs6962	SDHA	c.1969G>A p.Val657Ile	16	20	4	35	11	12.98% 796 hom		35	Blood		749	1851	40.6
154												3	Blood		1210	2213	54.7
155												107	Blood		251	524	48
156												122	Blood		548	1523	36
157												291	Blood		540	1445	37.4
158												118	Blood		888	2260	39.3
159												100	Blood		533	1886	28.3
160												50	Blood		797	2049	38.9
161												175	Frozen		184	526	35
162	24	rs1042446	SDHA	c.1974G>C p.Pro658Pro	0	0	0	0	0	2.348 % 0 hom		24	Blood		758	2090	36.3
163												88	Blood		440	1496	29.4
164												109	Blood		724	1966	36.9
165												224	Blood		926	2497	37.2
166												255	Blood		553	2360	23.5
167												291	Blood		343	1445	23.8
168												292	Blood		866	2598	33.4
169												297	Blood		481	1933	25
170												122	Blood		430	1518	28.4
171												128	Blood		359	1355	26.6
172												129	Blood		632	1681	37.7
173												167	Blood		718	2133	33.8
174												118	Blood		547	2256	24.3
175												100	Blood		830	1879	44.2
176												31	Blood		890	2223	40.1
177												14	Blood		665	1895	35.2
178	25	rs1042476	SDHA	c.*13T>C	0	0	0	0	0	0.2999% 0 hom	Positive SDHB-IHC in samples with ID 14,24, 88,	24	Blood		746	2093	36.1
179												100	Blood		837	1886	44.5
180												122	Blood		435	1523	28.6
181												128	Blood		363	1361	26.7
182												224	Blood		937	2505	37.4

183											128,129 and 291.	255	Blood		568	2362	24.1
184												291	Blood		344	1445	23.8
185												297	Blood		486	1933	25.2
186												167	Blood		738	2147	34.5
187												109	Blood		729	1971	37.1
188												88	Blood		449	1500	30
189												31	Blood		890	2223	40.1
190												14	Blood		670	1901	35.7
191	26	rs200769995	SDHA	c.*14G>A	0	0	0	0	0	0.2982% 0 hom	Positive SDHB-IHC in samples with ID 14,24,31, 100,109,128,129,167,224,291,292 and 297 confirms as a SNP	24	Blood		758	2091	36.3
192												109	Blood		724	3215	22.6
193												224	Blood		925	2502	37.1
194												255	Blood		561	2358	23.8
195												292	Blood		869	2598	33.5
196												297	Blood		482	1933	25
197												129	Blood		636	2846	22.4
198												167	Blood		737	3669	20.1
199												122	Blood		437	2592	16.9
200												100	Blood		827	3083	26.8
201												31	Blood		877	3681	23.9
202												14	Blood		674	1901	35.6
203	27	rs33927012	SDHB	c.487T>C p.Ser163Pro	1	1	0	0.2	2	1.254% 21 hom		27	Blood		2570	5000	51.6
204												28	Blood		25	38	65.8
205												109	Blood		1202	1210	99.8
206												107	Blood		713	1457	49
207												118	Blood		914	914	100
208												114	Blood		277	539	51.4
209	28	rs34261028	SDHB	c.424-19_424-14delTTCTTC	0	0	0	0	0	Not described	LARRIBA: POP-FT (ensembl) : 1/92 (1,1%)	122	Blood		258	758	37.1
210												11	Blood		588	1118	53.9
211	29	rs386134266	SDHB	c.424-19_424-14dupTTCTTC	0	0	0	0	0	Not described	Previously reported as an SNP in LOVD-	35	Blood		347	949	38.9
212												79	Blood		182	560	34.8
213												130	Blood		205	585	38.4

											Alrashdi (2010)						
214	30	rs148738139	SDHB	c.24C>T p.Ser8Ser	0	0	0	0	0	0.4299% 1 hom		150	Frozen		499	560	89.1
215												344	Blood		269	599	45.1
216	31	rs147815442	SDHB	c.21C>T p.Leu7Leu	0.05	0	0	0	0.13	0.05238 % 0 hom	Previously reported as an SNP in LOVD-Cascon (2002)	90	Blood		513	1018	50.5
217	32	rs2746462	SDHB	c.18C>A p.Ala6Ala	95	97	99	87	96	97.21 % 53297 hom		298	Blood		86	89	100
218												230	Blood		626	736	85.5
219												10	Genomip hi		418	699	60.1
220												11	Blood		971	1126	86.6
221												16	Blood		328	620	53.1
222												243	Blood		888	1270	70.4
223												40	Blood		756	894	85.1
224												117	Blood		1169	1157	99.9
225												100	Blood		407	407	100
226												107	Blood		696	713	98.6
227												122	Blood		737	740	100
228												128	Blood		1124	1130	99.8
229												129	Blood		902	909	99.9
230												167	Blood		1866	1877	99.9
231												31	Blood		1120	1122	100
232												224	Blood		504	507	99.8
233												255	Blood		1458	1473	99.5
234												291	Blood		845	859	99.4
235												292	Blood		1806	1814	99.8
236												297	Blood		689	701	99.7
237												52	Blood		468	842	55.8
238												14	Blood		684	694	99
239												88	Blood		1226	1230	99.7
240												3	Blood		287	292	99
241												62	Blood		1451	1451	100

242												218	Blood		589	715	82.4
243												24	Blood		1031	1046	99.3
244												86	Frozen		346	558	62.5
245												112	Blood		600	886	67.8
246												130	Blood		479	559	86.2
247												319	Blood		1001	1016	98.7
248	33	rs11203289	SDHB	c.8C>G p.Ala3Gly	1	0	0	4	0.13	0.436% 8 hom		33	Blood		24	50	48
249	34	rs77711105	RET	c.1942G>A p.Val648Ile	0	0	0	0	0	0.009076 % 0 hom	Previously reported as non pathogenic in ARUP-Cosci B (2011)	304	Genomip hi		268	521	51.8
250	35	rs148935214	RET	c.1946C>T p.Ser649Leu	0.09	0.28	0	0	0.13	0.03217 % 0 hom	Previously reported as an SNP in Erlic Z (2010)	116	Frozen		690	1316	52.5
251												345	Blood		182	337	54.3
252	36	rs1799939	RET	c.2071G>A p.Gly691Ser	15	23	10	9	20	20.33% 2840 hom		2	Blood		179	381	47
253												223	Blood		23	62	37.1
254												308	Blood		490	496	98.8
255												230	Blood		88	193	45.6
256												14	Blood		82	156	52.6
257												200	Blood		280	574	49
258												17	Blood		571	1176	48.6
259												239	Blood		251	571	44
260												20	Blood		150	306	49.5
261												241	Blood		233	597	39.2
262												242	Blood		26	46	56.5
263												243	Blood		276	560	49.7
264												24	Blood		151	270	56.1
265												291	Blood		70	153	45.8
266												245	Blood		204	421	48.8
267												247	Blood		462	466	99.1

268											249	Blood		39	90	43.3
269											256	Genomiphi		78	202	38.6
270											266	Blood		104	209	50.2
271											267	Blood		184	393	46.8
272											31	Blood		426	428	99.5
273											57	Blood		154	288	53.8
274											62	Blood		377	758	49.8
275											215	Blood		258	518	50
276											269	Blood		100	255	39.5
277											176	Frozen		93	201	46.3
278											179	Frozen		36	79	46.2
279											183	Frozen		57	227	25.1
280											184	Frozen		124	256	48.6
281											86	Frozen		534	1145	46.8
282											279	Blood		247	454	54.4
283											129	Blood		309	578	53.5
284											167	Blood		260	543	48.1
285											282	Blood		142	333	42.9
286											108	Blood		95	175	54.3
287											287	Blood		190	372	51.1
288											116	Frozen		445	949	47
289											344	Blood		125	264	47.9
290											345	Blood		122	278	44.4
291											349	Blood		154	326	47.2
292											350	Blood		160	310	52.1
293	37	rs1800861	RET	c.2307G>T p.Leu769Leu	72	77	49	90	76	74.19% 33769 hom		304	Genomiphi		574	1135	50.9
294												305	Blood		654	1351	48.6
295												4	Blood		201	384	53
296												225	Blood		341	737	46.5
297												5	Blood		262	476	55
298												6	Blood		78	141	55.3
299												11	Blood		290	575	50.6

300												13	Frozen		165	283	58.7
301												237	Blood		240	509	47.5
302												20	Blood		739	1457	50.8
303												24	Blood		1230	1260	98.5
304												244	Blood		529	997	53.5
305												245	Blood		613	1281	48.1
306												29	Blood		167	305	55.3
307												29	Frozen		152	258	58.9
308												248	Blood		773	1554	49.9
309												203	Blood		637	1245	51.2
310												251	Blood		724	1537	47.4
311												3	Blood		625	642	97.8
312												31	Blood		363	365	100
313												14	Blood		747	757	99.5
314												257	Blood		625	1229	51.3
315												262	Blood		435	896	48.9
316												62	Blood		432	434	100
317												100	Blood		257	257	100
318												266	Blood		573	1235	46.6
319												122	Blood		225	464	48.7
320												128	Blood		390	390	100
321												129	Blood		280	280	100
322												167	Blood		357	359	100
323												172	Blood		910	1846	49.6
324												109	Blood		180	341	52.9
325												57	Blood		170	340	50.3
326												117	Blood		234	112	47.9
327												224	Blood		502	502	100
328												255	Blood		913	929	99.1
329												291	Blood		897	913	98.9
330												297	Blood		550	564	98.4
331												107	Blood		671	1441	46.8
332												215	Blood		624	1182	52.8

333												270	Blood		910	1847	49.5
334												279	Blood		733	1528	48.2
335												282	Blood		287	638	45.3
336												118	Blood		419	419	100
337												88	Blood		417	418	99.8
338												288	Blood		758	1558	49
339												130	Blood		155	313	49.5
340												292	Blood		875	1725	50.9
341												294	Blood		111	227	49.3
342												352	Blood		840	1707	49.6
343	38	rs77724903	RET	c.2372A>T p.Tyr791Phe	0	0	0	0	0	0.1803% 1 hom	Toledo RA (2015) discarded pathogenicity of the variant	130	Blood		50,8	313	158
344												221	Blood		948	961	99.3
345												11	Blood		644	1086	59.3
346												237	Blood		82	148	55.4
347												19	Blood		12	50	24
348												122	Blood		335	549	61.1
349	39	rs1800862	RET	c.2508C>T p.Ser836Ser	3	5	0	2	5	4.666% 188 hom		244	Blood		513	1064	48.4
350												172	Blood		679	1548	44
351												270	Blood		661	1392	47.6
352												181	Frozen		369	777	47.6
353												278	Blood		14	44	31.8
354												279	Blood		456	956	47.7
355												2	Blood		218	459	47.5
356												223	Blood		95	195	48.7
357												230	Blood		246	543	45.6
358	40	rs1800863	RET	c.2712C>G p.Ser904Ser	16	22	10	11	20	0.003383 % 0 hom		14	Blood		174	337	51.9
359												17	Blood		502	1032	48.8
360												239	Blood		355	700	50.9
361												20	Blood		240	499	48.5
362												241	Blood		367	837	44.1

363												242	Blood		101	193	52.3
364												24	Blood		290	564	51.5
365												245	Blood		269	520	51.9
366												247	Blood		552	557	99.5
367												249	Blood		81	186	43.5
368												204	Blood		265	544	48.7
369												256	Genomiphi		94	159	59.1
370												44	Blood		215	419	51.3
371												266	Blood		289	521	55.7
372												267	Blood		320	674	47.5
373												172	Blood		292	606	48.3
374												62	Blood		472	1023	46.2
375												215	Blood		373	765	48.8
376												291	Blood		167	338	49.4
377												31	Blood		845	845	100
378												269	Blood		185	386	48.1
379												176	Frozen		562	977	57.6
380												179	Frozen		309	592	52.2
381												129	Blood		337	708	47.6
382												167	Blood		244	460	53
383												183	Frozen		94	357	26.3
384												184	Frozen		377	735	51.3
385												86	Frozen		494	1012	48.8
386												279	Blood		275	557	49.8
387												282	Blood		204	436	46.9
388												287	Blood		211	470	45.2
389												116	Frozen		783	1512	51.8
390												344	Blood		162	292	55.5
391												345	Blood		262	531	49.3
392												349	Blood		243	431	56.4
393												350	Blood		199	373	53.8

394	41	rs201389647	SDHAF2	c.37+17 T>C	0.23	0.28	0	1	0	Not described		218	Blood		16	33	48.5
395	42	rs2071313	MEN1	c.1269C>T p.Asp423Asp	31	33	40	5	40	39.34% 10125 hom		129	Blood		147	329	44.7
396												252	Blood		38	65	58.5
397	43	rs17849553, rs6720	MDH2	c.26C>T p.Ala9Val	51	41	62	72	33	44.54 % 1657 hom		72	Blood		518	992	52.22
398												116	Frozen		72	89	80.9
399												129	Blood	Low coverage region (E1-MDH2)	12	12	100
400												258	Blood		51	131	39.2
401												208	Blood	Low coverage region (E1-MDH2)	8	8	100
402												215	Blood	Low coverage region (E1-MDH2)	9	9	100
403												280	Blood		88	275	32.2
404												3	Blood	Low coverage region (E1-MDH2)	2	4	50
405												62	Blood		76	141	54.3
406												109	Blood		23	23	100
407												107	Blood		273	278	99.6
408												292	Blood		87	203	44.4
409												297	Blood	Low coverage region (E1-MDH2)	5	17	31.3
410												128	Blood		22	22	100

411												129	Blood	Low coverage region (E1-MDH2)	12	12	100
412												88	Blood		23	42	54.8
413												285	Blood		212	477	44.9
414												295	Blood	Low coverage region (E1-MDH2)	4	5	80
415												222	Blood	Low coverage region (E1-MDH2)			
416	44	rs79663210	MDH2	c.235+10G>A	7	5	2	5	11	8.615 % 528 hom		254	Blood		1537	3149	48.8
417												258	Blood		919	1868	49.3
418												270	Blood		1204	2461	49.1
419												297	Blood		1145	2349	48.9
420												109	Blood		1000	1008	99.6
421												128	Blood		960	964	99.6
422												280	Blood		1039	2008	51.9
423												127	Blood		370	808	45.8
424												129	Blood		385	745	51.7
425	45	rs11538801	MDH2	c.429G>A p.Pro143Pro	1	0.28	0	0.41	2	1.866% 38 hom		4	Blood		831	1604	51.9
426												64	Blood		736	1366	53.9
427												107	Blood		485	1106	44.2
428												77	Blood		1103	2127	52
429												285	Blood		845	1939	44.1
430												133	Frozen		891	1758	50.8
431	46	rs1637037	MDH2	c.633+17C>T	53	40	62	81	33	40.59% 11189 hom		11	Blood		454	953	47.6
432												16	Blood		215	446	48.3
433												258	Blood		311	630	49.4
434												208	Blood		278	519	53.7
435												62	Blood		385	765	50.3

436												215	Blood		306	600	51
437												72	Blood		1239	2487	49.82
438												292	Blood		416	811	51.4
439												297	Blood		251	511	49.2
440												76	Blood		365	754	48.7
441												88	Blood		805	806	99.9
442												280	Blood		296	588	50.6
443												103	Blood		201	315	63.8
444												3	Blood		128	228	56.1
445												100	Blood		665	666	100
446												117	Blood		585	278	47.5
447												107	Blood		441	449	98.7
448												109	Blood		784	790	99.5
449												285	Blood		486	985	49.4
450												116	Frozen		736	1364	54.1
451												127	Blood		499	1031	48.4
452												128	Blood		645	647	99.7
453												129	Blood		274	528	52
454												133	Frozen		760	1714	44.4
455												295	Blood		168	280	60.4
456	47	rs10256	MDH2	c.902A>G p.Lys301Arg	2	4	0	0.2	5	3.704 % 103 hom		231	Blood		604	1204	50.5
457												262	Blood		355	742	47.9
458												291	Blood		387	800	48.5
459												285	Blood		672	1507	44.7
460												117	Blood		752	395	52.6
461												122	Blood		258	473	54.5
462												112	Blood		303	653	46.5
463												220	Blood		455	837	54.6
464	48	rs3852673	TMEM127	c.621G>A p.Ala207Ala	11	13	8	5	18	16.34 % 1938 hom		1	Genomiphi		335	660	51
465												8	Genomiphi		102	229	44.7
466												8	Blood		244	545	44.9
467												19	Blood		175	293	59.7

468												261	Blood		389	850	46
469												207	Blood		348	693	50.2
470												172	Blood		358	726	49.4
471												214	Blood		1157	1165	99.8
472												71	Blood		28	46	63.6
473												75	Blood		169	240	70.4
474												109	Blood		275	500	55
475												272	Blood		92	168	54.8
476												175	Frozen		43	209	20.6
477												177	Frozen		188	383	49.6
478												278	Blood		65	98	66.3
479												24	Blood		268	608	44.4
480												255	Blood		294	623	47.4
481												291	Blood		272	623	43.7
482												292	Blood		590	1200	49.5
483												92	Blood		65	73	89
484												117	Blood		411	229	55.9
485												94	Blood		292	458	64.2
486												116	Frozen		561	881	63.8
487												342	Blood		274	530	51.9
488												345	Blood		340	735	46.3
489												348	Blood		321	645	49.8
490												351	Blood		222	456	48.8
491	49	rs189327749	TME M127	c.409+7C>T p.Ala207Ala	0.46	1	0	0	1	0.5177% 1 hom		230	Blood		767	1631	47.2
492	50	rs34677591	SDHD	c.34G>A p.Gly12Ser	1	2	0	0.2	1	0.7268 % 5 hom		32	Blood		33	82	40.2
493												69	Blood		582	1136	51.4
494												182	Frozen		265	709	37.5
495	51	rs11214077	SDHD	c.149A>G p.His50Arg	1	2	0	0	1	0.6515 % 6 hom		3	Blood		762	1623	47.1
496												224	Blood		711	1503	47.4
497												224	Blood		711	1503	47.4
498												60	Blood		1236	2403	51.5

499	52	rs9919552	SDHD	c.204C>T p.Ser68Ser	10	3	0	39	1	3.976% 622 hom		237	Blood		509	1149	44.6
500												21	Blood		325	838	38.8
501												32	Blood		467	1081	43.3
502												69	Blood		301	663	45.7
503												83	Blood		238	575	41.8
504												100	Blood		282	600	47.1
505	53	rs35215598, rs75726722	SDHC	c.20+9_20+10insGT	14	8	22	23	5	10.53 % 891 hom		100	Blood		73	125	58.4
506												134	Frozen		186	330	56.4
507	54	rs61737760	FH	c.927G>A p.Pro309Pro	3	2	4	2	3	3.489 % 104 hom		1	Genomiphi		1018	1998	51.3
508												9	Blood		951	1972	48.3
509												201	Blood		679	1327	51.2
510												239	Blood		808	1572	51.5
511												264	Blood		938	1812	52.1
512												267	Blood		757	1559	48.7
513												70	Blood		750	1544	49.1
514												98	Blood		784	1520	51.8

PANEL-II

MUTATIONS

MUT.	ID	Type of sample	Final	Gene	Alt Read Depth	Read Depth	Alt Variant Freq	Consequence	cDNA	Protein
1	78	FFPE	Control germline mutation SDHA	SDHA	30	32	93.75	missense	c.1754G>A	p.Arg585Gln
2	411	FFPE	Control somatic mutation HRAS	HRAS	638	2266	28.16	missense	c.182A>G	p.Gln61Arg
3	419	FFPE	Control somatic mutation HRAS	HRAS	1248	3063	40.74	missense	c.37G>C	p.Gly13Arg
4	396	FFPE	Control somatic mutation VHL	VHL	19	71	26.76	missense	c.227T>A	p.Phe76Tyr
					18	67	26.87	synonymous	c.228C>T	p.Phe76Phe
5	422	FFPE	Control somatic mutation VHL	VHL	50	338	14.79	missense	c.260T>C	p.Val87Ala
6	454	FFPE	Control somatic mutation VHL	VHL	196	851	23.03	missense	c.482G>A	p.Arg161Gln
7	453	FFPE	Control germline mutation SDHB (c.595delTACTGGTGGAinsGG; p.Tyr199Glyfs*19)	SDHB	393	1391	28.25	frameshift	c.605delA	p.Asn202Thrfs*18
					403	1129	35.7	frameshift	c.601_604delTGGA	p.Trp201Thrfs*18
					390	1322	29.5	frameshift	c.595_601delTACTGGT	p.Tyr199Glyfs*19
					410	1331	30.8	frameshift	c.595_598delTACT	p.Tyr199Glyfs*20

8	380	FFPE	Control germline mutation RET	RET	45	81	55.56	missense	c.1900T>C	p.Cys634Arg
9	386	FFPE	Control somatic mutation RET	RET	29	106	27.36	missense	c.2753T>C	p.Met918Thr
10	122	FFPE	Control somatic mutation EPAS1	EPAS1	1666	6262	26.6	missense	c.1591C>G	p.Pro531Ala
11	297	FFPE	Control germline mutation SDHAF2	SDHAF2	7526	12709	59.22	stop_gained	c.362G>A	p.Trp121*
12	416	FFPE	Control germline mutation MDH2	MDH2	195	359	54.32	splice_donor	c.429+1G>T	
13	117	FFPE	Control somatic mutation SDHD (VUS SDHD)	SDHD	1188	1802	65.93	stop_gained	c.112C>T	p.Arg38*

VARIANTS OF UNKNOWN SIGNIFICANCE (VUS)

VUS	ID	Type of sample	Final	Gene	Alt Read Depth	Read Depth	Alt Variant Freq	Consequence	cDNA	Protein
1	382	FFPE	Control VUS germline MAX	MAX	67	126	53.17	missense	c.425C>T	p.Ser142Leu

POLYMORPHISMS (SNPs)

rs	Unique rs	dbSNP ID	Gene	HGVSc HGVSp	Allele Freq	Allele Freq Amr	Allele Freq Asn	Allele Freq Af	Allele Freq Eur	Allele Freq ExAC		ID		Alt Read Depth	Read Depth	Alt Variant Freq
1	1	rs34635677	SDHA	c.113A>T p.Asp38Val	1	1	0	0	3	3.529% 103 hom		109		1342	2759	48.64
2												255		503	939	53.57
3	2	rs1139424	SDHA	c.309A>G p.Ala103Ala	17	20	4	42	9	15.29% 2023 hom		62		306	681	44.93
4												291		253	691	36.61
5	3	rs6555055	SDHA	c.619A>C p.Arg103Arg	22	23	5	56	11	15.68% 2372 hom		100		1079	2698	39.99
6												62		1526	3549	43
7												122		1621	3979	40.74
8												291		1217	2804	43.4
9												3		1245	2511	49.58
10												107		1369	2711	50.5
11												118		2024	5649	35.83
12												88		639	1267	50.43
13	4	rs2115272	SDHA	c.684T>C p.Asn228Asn	22	23	5	56	11			100		688	1556	44.22
14												62		598	1075	55.63

15										15.65% 2368 hom		122		642	1320	48.64
16												291		711	1172	60.67
17												3		466	914	50.98
18												107		477	1094	43.6
19												118		247	847	29.16
20												88		219	463	47.3
21												100		527	527	100
22												62		819	843	97.15
23												117		282	1024	27.54
24												122		754	758	99.47
25												109		294	294	100
26												129		514	514	100
27												31		1278	1284	99.53
28												291		423	423	100
29												3		479	487	98.36
30												297		1087	2562	42.43
31	5	rs2288461	SDHA	c.771-11A>G	86	88	69	99	88	88.10 % 47377 hom		108		1118	1125	99.38
32												24		82	224	36.61
33												107		735	738	99.59
34												118		481	1647	29.2
35												128		561	561	100
36												14		110	110	100
37												167		1194	1198	99.67
38												88		227	227	100
39												224		567	1435	39.51
40												255		399	399	100
41												292		90	103	87.38
42	6	rs34771391	SDHA	c.822C>T p.Gly274Gly	1	1	0	2	0.13	0.4620% 3 hom		128		561	565	99.29
43												100		1282	3190	40.19
44	7	rs1126417	SDHA	c.891T>C p.Pro297Pro	63	70	33	73	75	70.78% 31200 hom		62		3016	6336	47.6
45												117		2377	4769	49.84
46												122		6787	6816	99.57

47												109		4175	4294	97.23
48												129		723	2669	27.09
49												31		3582	6981	51.31
50												291		4131	4272	96.7
51												3		4929	4996	98.66
52												297		5887	11684	50.39
53												108		5980	5984	99.93
54												24		1084	2325	46.62
55												107		2080	4045	51.42
56												118		2406	7321	32.86
57												128		5439	5493	99.02
58												14		1342	1404	95.58
59												167		6283	6323	99.37
60												88		3326	3337	99.67
61												224		4731	9334	50.69
62												255		2386	4502	53
63												292		1449	1605	90.28
64	8	rs7710005	SDHA	c.896-20A>G	22	23	5	55	11	15.66% 2369 hom		62		3484	6020	57.87
65												122		3591	7128	50.38
66												291		2313	4541	50.94
67												107		3848	8360	46.03
68												118		2119	6691	31.67
69												88		2381	5063	47.03
70	9	rs142849100	SDHA	c.969C>T p.Gly323Gly	0.23	1	0	0	0.4	0.7017% 12 hom		255		256	598	42.81
71	10	rs1041949	SDHA	c.1038C>G p.Ser346Ser	22	24	5	59	11	15.97 % 2574 hom		100		521	1004	51.89
72												62		532	1205	44.15
73												122		795	1536	51.76
74												291		465	1089	42.7
75												3		348	838	41.53
76												107		631	1197	52.72
77												118		486	1361	35.71
78												88		229	413	55.45

79	11	rs35277230	SDHA	c.1170C>T p.Phe390Phe	9	3	0	36	0.13	3.24% 579 hom		100		239	415	57.59
80												3		414	746	55.5
81	12	rs150831951	SDHA	c.1305G>T p.Leu435Leu	1	2	0	0.2	3	1.927% 40 hom		128		4339	6185	70.15
82	13	rs34779890	SDHA	c.1413C>T p.Ile471Ile	0.14	0.28	0	0.2	0.13	0.2199% 1 hom		128		6814	6831	99.75
83	14	rs10039029	SDHA	c.1680G>A p.Thr560Thr	21	23	4	53	11	15.27 % 1873 hom		62		396	1697	23.34
84												122		701	3379	20.75
85												291		694	2814	24.66
86												3		551	2335	23.6
87												107		404	1901	21.25
88												118		703	4637	15.16
89												88		236	1221	19.33
90	15	rs77210621	SDHA	c.1752A>G p.Ala584Ala	22	23	4	56	11	15.59 % 2311 hom		100		1094	2411	45.38
91												62		2190	3977	55.07
92												122		1511	2968	50.91
93												291		2110	4106	51.39
94												3		1208	2634	45.86
95												107		1341	2525	53.11
96												118		1454	4030	36.08
97												88		628	1272	49.37
98	16	rs35549341	SDHA	c.1908+15C>T	5	7	0.17	1	11	Not described		122		476	484	98.35
99												109		287	613	46.82
100												297		951	1909	49.82
101												167		700	1319	53.07
102	17	rs372662724	SDHA	c.1909-14_1909-13delCT	0	0	0	0	0	0.0008255% 0 hom	Positive SDHB-IHC confirms as a SNP	291		164	717	22.87
103	18	rs6961	SDHA	c.1932G>A p.Val644Val	34	32	33	58	20	17.16% 2406 hom		100		1402	1817	77.16
104												62		410	793	51.7
105												122		1561	2319	67.31
106												109		615	1566	39.27

107												129		815	1408	57.88
108												31		595	1546	38.49
109												291		568	719	79
110												3		442	753	58.7
111												297		690	3046	22.65
112												24		263	648	40.59
113												107		455	1130	40.27
114												118		713	1293	55.14
115												128		221	1369	16.14
116												14		168	506	33.2
117												167		958	2606	36.76
118												88		375	574	65.33
119												224		708	2381	29.74
120												255		201	719	27.96
121												292		81	425	19.06
122	19	rs148627127	SDHA	c.1944_1945delTT p.Leu649Glufs*4	3	3	6	3	1	1.35 % 0 hom		100		360	1829	19.68
123												122		475	2329	20.4
124												297		683	3085	22.14
125	20	rs6962	SDHA	c.1969G>A p.Val657Ile	16	20	4	35	11	12.98 % 796 hom		100		718	1821	39.43
126												122		1063	2324	45.74
127												291		369	726	50.83
128												3		444	751	59.12
129												107		460	1141	40.32
130												118		296	1288	22.98
131	21	rs1042446	SDHA	c.1974G>C p.Pro658Pro	0	0	0	0	0	2.348 % 0 hom		100		657	1818	36.14
132												122		492	2327	21.14
133												109		612	1574	38.88
134												129		794	1390	57.12
135												31		587	1550	37.87

136												291		187	723	25.86
137												297		670	3072	21.81
138												24		256	650	39.38
139												118		388	1279	30.34
140												128		221	1372	16.11
141												14		158	507	31.16
142												167		938	2595	36.15
143												88		79	575	13.74
144												224		705	2383	29.58
145												255		191	721	26.49
146												292		55	419	13.13
147	22	rs1042476	SDHA	c.*13T>C	0	0	0	0	0	0.2999% 0 hom	Positive SDHB-IHC in samples with ID 14,24, 88, 128,129 and 291.	291		20	112	17.86
148												24		93	288	32.29
149												128	Low coverage region (E1-SDHA)	12	162	7.41
150												14		61	238	25.63
151												88		28	279	10.04
152												255		77	340	22.65
153	23	rs200769995	SDHA	c.*14G>A	0	0	0	0	0	0.2982% 0 hom	Positive SDHB-IHC in samples with ID 14,24,31, 100,109,128,129,167,224,291,292 and 297 confirms as a SNP	100	Low coverage region (E1-SDHA)	193	4089	4.72
154												109	Low coverage region (E1-SDHA)	254	4263	5.96
155												129	Low coverage region (E1-SDHA)	252	3396	7.42
156												31		806	5197	15.51
157												297	Low coverage region (E1-SDHA)	453	8968	5.05
158												24		258	1810	14.25
159												14		183	1162	15.75
160												167	Low coverage region (E1-SDHA)	530	5356	9.9
161												224	Low coverage region (E1-SDHA)	610	6414	9.51

162												255	Low coverage region (E1-SDHA)	187	2717	6.88
163												292	Low coverage region (E1-SDHA)	76	1415	5.37
164	22 and 23	rs1042476 and rs200769995	SDHA	c.*13_*14invTG	0	0	0	0	0	Not described	Positive SDHB-IHC in samples 31,100,109,122,167 and 224 confirms as a SNP.	100		651	1806	36.05
165												122		417	2289	18.22
166												109		581	1556	37.34
167												31		565	1542	36.64
168												297		255	1487	17.15
169												167		361	1209	29.86
170												224		275	1127	24.4
171	24	rs63650860	VHL	c.183C>G p.Pro61Pro	0	0	0	0	0.05	0.2542% 0 hom	Previously reported as a SNP in Gallou (1999)	412		275	358	76.82
172	25	rs33927012	SDHB	c.487T>C p.Ser163Pro	1	0	0.2	2	0.95	1.254 % 21 hom		107		318	689	46.15
173	26	rs386134266	SDHB	c.424-19_424-14dupTTCTTC	0	0	0	0	0	Not described	Previously reported as a SNP in Rattenberry (2013)	122	Positive SDHB-IHC	316	1625	19.45
174	27	rs2746462	SDHB	c.18C>A p.Ala6Ala	97	99	87	96	95.15	97.21 % 53297 hom		100		1220	1225	99.59
175												62		1192	1215	98.11
176												117		952	954	99.79
177												122		1447	1476	98.04
178												109		1858	1866	99.57
179												129		578	578	100
180												31		1248	1248	100
181												291		1255	1263	99.37
182												3		536	538	99.63
183												297		1626	1636	99.39
184												108		960	963	99.69

185												24		207	207	100
186												107		462	462	100
187												118		1494	1506	99.2
188												128		1016	1051	96.67
189												14		238	240	99.17
190												167		1256	1258	99.84
191												88		164	164	100
192												224		746	748	99.73
193												255		368	408	90.2
194												292		177	177	100
195	28	rs1799939	RET	c.2071G>A p.Gly691Ser	23	10	9	20	15.7	2033% 2840 hom		379		552	1151	47.96
196												381		666	1480	45
197												385		313	728	42.99
198												62		674	1346	50.07
199												404		307	766	40.08
200												410		713	1366	52.2
201												129		360	763	47.18
202												413		427	968	44.11
203												31		1409	1415	99.58
204												428		554	897	61.76
205												291		467	1105	42.26
206												451		313	434	72.12
207												382		201	405	49.63
208												108		783	1433	54.64
209												399		321	668	48.05
210												24		177	324	54.63
211												14		121	210	57.62
212												167		795	1071	74.23
213	29	rs1800861	RET	c.2307G>T p.Leu769Leu	77	49	90	76	80.26	74.19 % 33769 hom		379		1406	2917	48.2
214												100		2319	2327	99.66
215												62		2191	2227	98.38
216												117		1291	2163	59.69
217												122		2404	4835	49.72

218												410		1907	3467	55
219												109		1628	3311	49.17
220												411		1077	2100	51.29
221												129		2158	2169	99.49
222												412		1097	3304	33.2
223												419		1000	2207	45.31
224												31		3028	3041	99.57
225												291		2942	3033	97
226												434		1135	2227	50.97
227												3		2584	2588	99.85
228												297		4331	4343	99.72
229												108		3333	3344	99.67
230												24		1395	1436	97.14
231												405		600	1097	54.69
232												107		1153	2285	50.46
233												118		4521	4531	99.78
234												128		2429	2475	98.14
235												14		1069	1114	95.96
236												416		2328	4373	53.24
237												167		2428	2457	98.82
238												88		1112	1142	97.37
239												224		3440	3448	99.77
240												255		1623	1752	92.64
241												292		246	805	30.56
242	30	rs1800862	RET	c.2508C>T p.Ser836Ser	5	0	2	5	4.24	4.666% 188 hom		379		2116	4346	48.69
243												122		1809	3541	51.09
244												410		2060	3963	51.98
245												412		1012	1937	52.25
246												414		951	2043	46.55
247												416		3639	7029	51.77
248	31	rs1800863	RET	c.2712C>G p.Ser904Ser	22	10	11	20	16.09	20.57 % 2745 hom		379		491	1021	48.09
249												381		817	1591	51.35
250												62		367	806	45.53

251												404		198	491	40.33
252												410		636	1326	47.96
253												129		473	912	51.86
254												413		408	909	44.88
255												31		1033	1038	99.52
256												291		274	642	42.68
257												451		371	805	46.09
258												382		201	257	78.21
259												108		690	1430	48.25
260												399		421	770	54.68
261												24		153	233	65.67
262												14		57	168	33.93
263												167		960	1309	73.34
264												421		410	655	62.6
265	32	rs370174263	SDHAF2	c.451C>G p.Gln151Glu	0	0	0	0	0.01	Not described	Positive SDHB-IHC confirms as a SNP.	395		296	835	35.45
266												292		144	213	67.61
267												451		122	361	33.8
268												3		303	703	43.1
269												297		422	968	43.6
270												385		460	1018	45.19
271												62		626	1121	55.84
272												107		316	316	100
273	33	rs17849553, rs6720	MDH2	c.26C>T p.Ala9Val	41	62	72	33	39.15	44.54% 1657 hom		109		2402	2458	97.72
274												129		333	636	52.36
275												128		1175	1193	98.49
276												88		112	204	54.9
277												377	Low coverage region (E1-MDH2)			
278												412	Low coverage region (E1-MDH2)			

279	34	rs79663210	MDH2	c.235+10G>A	5	2	5	11	8.99	8.615% 528 hom		297		1756	3250	54.03
280												385		889	1494	59.5
281												109		1779	1781	99.89
282												129		720	1254	57.42
283												128		1599	1627	98.28
284	35	rs11538801	MDH2	c.429G>A p.Pro143Pro	0.28	0	0.41	2	1.16	1.866% 38 hom		107		65	111	58.56
285												412		88	158	55.7
286	36	rs1637037	MDH2	c.633+17C>T	40	62	81	33	44.02	40.59% 11189 hom		3		2460	4798	51.27
287												297		5726	12555	45.61
288												385		1692	3365	50.28
289												100		2629	2699	97.41
290												62		1938	3806	50.92
291												396		965	1982	48.69
292												117		2148	4110	52.26
293												107		3770	3782	99.68
294												109		4032	4052	99.51
295												129		1562	3005	51.98
296												412		2952	7192	41.05
297												128		4638	4714	98.39
298												88		1684	3194	52.72
299												292		636	1484	42.86
300												451		1772	3478	50.95
301	37	rs10256	MDH2	c.902A>G p.Lys301Arg	4	0	0.2	5	3.6	3.704% 103 hom		389		544	1205	45.15
302												117		808	1631	49.54
303												122		891	1453	61.32
304												291		688	1383	49.75
305	38	rs3852673	TMEM127	c.621G>A p.Ala207Ala	13	8	5	18	13.87	16.34% 1938 hom		381		897	1739	51.58
306												117		754	1251	60.27
307												410		902	1883	47.9
308												109		390	870	44.83
309												291		660	1083	60.94
310												24		148	294	50.34
311												405		143	279	51.25

312												255		134	398	33.67
313												292		147	373	39.41
314	39	rs11214077	SDHD	c.149A>G p.His50Arg	2	0	0	1	0.63	0.6515% 6 hom		378		520	1271	40.91
315												389		643	1282	50.16
316												3		757	1522	49.74
317												405		319	621	51.37
318												224		337	1684	20.01
319	40	rs9919552	SDHD	c.204C>T p.Ser68Ser	3	0	39	1	12.01	3.976% 622 hom		384		181	482	37.55
320												100		422	878	48.06
321	41	rs35215598, rs75726722	SDHC	c.20+11_20+12dupTG	8	22	23	5	11.22	10.53% 891 hom		100		335	923	36.29
322												411		296	653	45.33
323	42	rs61737760	FH	c.927G>A p.Pro309Pro	2	4	2	3	2.21	3.489 % 104 hom		108		1279	2490	51.37

Supplementary table S6. Variants (mutations and VUS) found by TGPs and validated by Sanger sequencing. Mut.:Mutation; Mut$^{u:}$ Unique mutation; IHC: SDHB-immunohistochemistry; M or VUS: mutation or Variant of Unknown Significance; ExAC database: Prevalence described in the The Exome Aggregation Consortium (ExAC); LOVD: Presence described in the Leiden Open (source) Variation Database; ND: not described; Pubmed: Previously published; Alt.: Altered; FFPE: Formalin fixed paraffin-embedded tumor tissue; Neg.: Negative SDHB-IHC; Pos: Positive SDHB-IHC; SM: Somatic mutations, GM: Germline mutation; Tumor, no blood: Mutation found in tumor DNA sample, and no germline DNA available to check if the variant is somatic or germline; SIFT/Polyphen: protein functional prediction in SIFT and Polyphen 2; tol.: tolerated; del.:deleterious; COSMIC: prevalence in COSMIC: Catalogue of Somatic Mutations in Cancer; AF: Allele Frequency; Amr: Americans; Asn: Asian; Eur: European population; LOHLoss Of Heterozygosity; qPCR: quantitative PCR; RBP1: Retinol Binding Protein 1; 5hmC: 5-hydroxymethylcytosine; 2SC: 2-Succinocysteine.

Mut	Mut^{u}	M or VUS	Gene	cDNA	Protein	ExAC database	LOVD	Pubmed	ID	Sample	IHC	Alt Read Depth	Read Depth	Alt Variant Freq	Type
1	1	M	*SDHA*	c.91C>T	p.Arg31*	20 of 121408 allele count. 0 homozygotes. 0.0001647 allele frequency.	Reported 2 times as pathogenic (r.1065_1260del (exon 9 skipping)) in Netherlands: Nijmegen	Previously reported Korpershoek (2011) as pathogenic (Neg. SDHB/SDHA-IHC)	368	Blood	Neg.	754	1643	46.1	GM
2	2	M	*SDHA*	c.1334C>T	p.Ser445Leu	ND	ND	Previously reported Papathomas (2015) as pathogenic (Neg. SDHB/SDHA-IHC)	124	Blood	Neg.	834	1538	54.3	GM
3	3	M	*HRAS*	c.182A>G	p.Gln61Arg	ND	ND	Previously reported Crona (2013)	88	FFPE	Pos.	425	934	45.5	SM
										Blood					
4		M	*HRAS*	c.182A>G	p.Gln61Arg	ND	ND	Previously reported Crona (2013)	151	Frozen	Pos.	15	24	62.5	SM
5		M	*HRAS*	c.182A>G	p.Gln61Arg	ND	ND	Previously reported Crona (2013)	359	Frozen	ND	116	313	37.1	SM
6		M	*HRAS*	c.182A>G	p.Gln61Arg	ND	ND	Previously reported Crona (2013)	360	Frozen	ND	55	185	29.9	SM
7		M	*HRAS*	c.182A>G	p.Gln61Arg	ND	ND	Previously reported Crona (2013)	376	Frozen	ND	25	210	11.9	SM
8		M	*HRAS*	c.182A>G	p.Gln61Arg	ND	ND	Previously reported Crona (2013)	433	FFPE	Pos.	470	1961	23.97	SM
9		M	*HRAS*	c.182A>G	p.Gln61Arg	ND	ND	Previously reported Crona (2013)	436	FFPE	Pos.	1006	2266	44.4	SM
10	4	M	*HRAS*	c.182A>T	p.Gln61Leu	ND	ND	ND. but reported p.Gln61Arg in Crona (2013) and p.Gln61Lys in Oudijk (2013)	449	FFPE	Pos.	222	1272	17.45	SM
11	5	M	*HRAS*	c.37G>C	p.Gly13Arg	ND	ND	Previously reported Crona (2013)	320	Frozen	ND	353	911	38.8	SM

12		M	*HRAS*	c.37G>C	p.Gly13Arg	ND	ND	Previously reported Crona (2013)	375	Frozen	ND	55	345	16.1	SM
13	6	M	*VHL*	c.193T>G	p.Ser65Ala	ND	ND	VHL alliance database: Previously reported in Neumann (2002) in VHL disease and in Burnichon (2011) in PPGL; UMD-VHL: ND. but reported p.Ser65Pro; p.Ser65Trp and p.Ser65Leu	148	Frozen	Pos.	81	192	44.8	SM
14		M	*VHL*	c.193T>G	p.Ser65Ala	ND	ND	VHL alliance database: Previously reported in Neumann (2002) in VHL disease and in Burnichon (2011) in PPGL; UMD-VHL: ND. but reported p.Ser65Pro; p.Ser65Trp and p.Ser65Leu	153	Frozen	Pos.	48	172	29.1	SM
16		M	*VHL*	c.193T>G	p.Ser65Ala	ND	ND	VHL alliance database: Previously reported in Neumann (2002) in VHL disease and in Burnichon (2011) in PPGL; UMD-VHL: ND. but reported p.Ser65Pro; p.Ser65Trp and p.Ser65Leu	372	Frozen	ND	9	67	13.6	?
15	7	M	*VHL*	c.193T>A	p.Ser65Thr	ND	ND	Previously reported in Crona (2014); UMD-VHL: ND. but reported p.Ser65Pro; p.Ser65Trp and p.Ser65Leu	190	Frozen	ND	36	135	27.7	SM
										Blood					
17	8	M	*VHL*	c.233A>G	p.Asn78Ser	ND	ND	VHL alliance database: Previously reported in Chen (1995); UMD-VHL: Reported 6 times	326	Frozen	ND	30	52	57.7	SM
18	9	M	*VHL*	c.244C>G	p.Arg82Gly	ND	ND	Previously reported in Burnichon (2011); UMD-VHL: ND. but described p.Arg82Cys and p.Arg82Pro	291	FFPE	Pos.	194	283	68.55	SM
										Blood					
19	10	M	*VHL*	c.376G>A	p.Asp126Asn	ND	ND	VHL alliance database: Previously reported in Brauch (2004); UMD-VHL: ND. but reported p.Asp126Gly	355	Frozen	ND	577	885	65.6	?
20	11	M	*VHL*	c.407T>G	p.Phe136Cys	ND	ND	VHL alliance database: Previously reported in Whaley (1994); UMD-VHL: Reported 1 time	323	Frozen	ND	255	1892	13.6	SM
21	12	M	*VHL*	c.414A>G	p.Pro138Pro	ND	ND	Previously reported in A. Giménez Roqueplo P11.242 European-society-of-human-genetics Meeting; UMD-VHL: ND	182	Frozen	Pos.	239	913	26.4	SM
22		M	*VHL*	c.414A>G	p.Pro138Pro	ND	ND	Previously reported in A. Giménez Roqueplo P11.242 European-society-of-human-genetics Meeting; UMD-VHL: ND	353	Frozen)	ND	227	716	31.7	SM
23	13	M	*VHL*	c.464T>G	p.Val155Gly	ND	ND	UMD-VHL: ND. but reported p.Val155Met and p.Val155Leu	274	Frozen	ND	25	179	14	SM
24	14	M	*VHL*	c.494T>G	p.Val165Gly	ND	ND	VHL alliance database: Previously reported in Baker (2000); UMD-VHL: ND	100	FFPE	Pos.	58	1240	4.68	SM
										Blood					
25	15	M	*VHL*	c.500G>A	p.Arg167Gln	ND	ND	VHL alliance database: Previously reported in Crossey (1994); UMD-VHL: Reported 28 times	327	Frozen	ND	194	458	42.5	SM

26	16	M	*VHL*	c.598C>T	p.Arg200Trp	ND	Reported 2 times as pathogenic	VHL alliance database: Previously reported in Kishida. Stackhouse et al. (1995); UMD-VHL: Reported 3 times	374	Blood	ND	253	515	49.1	GM
27	17	M							125	Blood	Pos.				SM
			VHL	c.284C>G	p.Pro95Arg	ND	ND	VHL alliance database: Previously reported in Gallou (1999)		FFPE		123	256	48.0	
28	18	M	*SDHB*	c.649C>T	p.Arg217Cys	ND	Reported 2 times (somatic mutation in 1)	Previously described Burnichon (2009)	149	Frozen	Neg.	315	404	78	GM
29		M	*SDHB*	c.649C>T	p.Arg217Cys	ND	Reported 2 times (somatic mutation in 1)	Previously described Burnichon (2009)	334	Frozen	ND	241	375	64.4	GM
30	19	M	*SDHB*	c.591delC	p.Ser198Alafs*22	ND	Reported 2 times	Previously described Burnichon (2009)	162	Blood	Neg.	252	607	41.7	GM
31	20	M	*SDHB*	c.503dupA	p.Gln169Alafs*10	ND	ND		329	Frozen	ND	1158	1452	80.2	GM
32		M	*SDHB*	c.503dupA	p.Gln169Alafs*10	ND	ND		331	Frozen	ND	769	971	79.6	GM
33	21	M	*SDHB*	c.424-3C>G		ND	Reported 1 time: Proven splice defect: United States. exon 5 skipping and truncation at 248aa. Spain	Previously reported Papathomas (2015) as pathogenic (Neg. SDHB/SDHA-IHC)	2	Blood	Neg.	345	738	46.7	GM
34	22	M	*SDHB*	c.393dupA	p.His132Thrfs*23	ND	ND	ND, but previously reported Maier-Woelfle (2004) as pathogenic c.395A>C; p.His132Pro	441	Blood	Neg.	2591	5000	52.0	GM
35	23	M	*SDHB*	c.380T>G	p.Ile127Ser	ND	Reported 1 time: concluded pathogenicity: unknown	Previously reported Papathomas (2015) as pathogenic (Neg. SDHB/SDHA-IHC)	371	Frozen	ND	1003	1667	60.3	? (probably GM)
36	24	GD	*SDHB*	exon 1 deletion		ND	Reported 2 times	Previously reported in Cascon (2006)	152	Frozen	ND				GM
37	25	M	*NF1*	c.349delA	p.Ile117Serfs*48	ND	ND	Previously reported in Pros (2008) in NF1 patient	365	Frozen	ND	264	365	72.3	? (probably SM. LOH)
38	26	M(X2)	*NF1*	c.517G>C	p.Asp173His	ND	ND		434	FFPE	Pos.	410	893	45.91	SM
	27		*NF1*	c.519delT	p.Asp173Glufs*5	ND	ND					410	892	45.96	
39	28	M	*NF1*	c.574C>T	p.Arg192*	1 of 119128 allele count. 0 homozygotes. 0.000008394 allele frequency.	Reported 23 times as pathogenic	Previously described in Messiaen (2000)	135	Frozen	Pos.	339	522	64.9	SM
40	29	M	*NF1*	c.654+1G>A		ND	Reported 2 times as pathogenic	ND, but previously reported in Laycock-van Spyk (2011) in a case with NF1 the variant c.654+1G>T as pathogenic	330	Frozen	ND	898	1070	83.9	SM
41	30	M	*NF1*	c.889-1G>T		ND	ND, but c.889-1G>C reported as pathogenic in Netherlands: Rotterdam	ND, but reported in Laycock-van Spyk (2011) as pathogenic in NF1 c.889-2A>G	385	FFPE	Pos.	659	1094	60.24	SM
42	31	M	*NF1*	c.901_909delGACAGTCTA	p.Asp301_leu303del	ND	ND		407	FFPE	Pos.	758	1519	49.9	SM

43	32	M	*NF1*	c.980del T	p.Leu327Argfs*49	ND	ND		409	FFPE	Pos.	1117	1996	55.96	SM
44	33	M	*NF1*	c.1607C >G	p.Ser536*	ND	Reported as pathogenic in Netherlands: Rotterdam	ND, but previously reported in Messiaen (2000) as pathogenic the variant c.1607 C>A; P.Ser536*	194	Frozen	ND	1505	1725	87.4	SM
										Blood					
45		M	*NF1*	c.1607C >G	p.Ser536*	ND	Reported as pathogenic in Netherlands: Rotterdam	ND, but previously reported in Messiaen (2000) as pathogenic the variant c.1607 C>A; P.Ser536*	195	Frozen	ND	808	830	97.6	SM
										Blood					
46	34	M	*NF1*	c.1642-1G>A		ND	Reported as pathogenic in Netherlands: Rotterdam		313	Frozen	ND	148	221	67.6	SM
47	35	M	*NF1*	c.1706_1707insAT	p.Phe570Tyrfs*17	ND	ND		325	Frozen	ND	108	179	61	GM
48	36	M	*NF1*	c.2125T >C	p.Cys709Arg	ND	Reported as pathogenic in Netherlands: Rotterdam		388	FFPE	Pos.	73	343	21.28	SM
49	37	M	*NF1*	c.2364_2385delAAAGCTAATCCTTAACTATCCA	p.Leu790Profs*24	ND	ND		381	FFPE	Pos.	764	889	85.94	SM
50	38	M	*NF1*	c.2464G >T	p.Gly822*	ND	Reported as pathogenic in Netherlands: Rotterdam	Previously reported in Bausch (2007) in patients with NF1 and PCC	357	Blood	ND	220	485	45.5	GM
51	39	M	*NF1*	c.2592_2593delCC	p.Pro865Thrfs*7	ND	ND		447	FFPE	Pos.	1401	2048	68.41	? (probably SM. LOH)
52	40	M	*NF1*	c.2666delC	p.Thr889Asnfs*13	ND	ND	Previously reported in Fahsold (2000) in NF1	404	FFPE	Pos.	1628	2774	58.69	SM
53	41	M	*NF1*	c.2703delA	p.Met902Trpfs*22	ND	ND		430	FFPE	Pos.	6147	7940	77.42	? (probably SM. LOH)
54	42	M	*NF1*	c.3114-1delG	p.Asn1039Ilefs*4	ND	ND		129	FFPE	Pos.	1001	2010	49.8	SM
										Blood					
55	43	M	*NF1*	c.3132C >A	p.Tyr1044*	ND	ND		176	Frozen	ND	146	151	98.6	SM
56	44	M	*NF1*	c.3783_3787delTTCTA	p.Phe1261Leufs*21	ND	ND		133	Frozen	Pos.	860	1145	75.2	SM
57	45	M	*NF1*	c.5609+1G>A		ND	ND		424	FFPE	Pos.	842	2131	39.51	SM

58	46	M	*NF1*	c.6236delC	p.Ala2079Valfs*3	ND	ND		332	Frozen	ND	769	931	82.6	GM
59	47	M	*NF1*	c.6585_6586dupGA	p.Thr2196Argfs*5	1 (Latino) of 121402 allele count. 0 homozygotes. 0.000008237 allele frequency.	ND		392	FFPE	Pos.	659	2035	32.38	SM
60	48	M	*NF1*	c.6854_6855insT	p.Asn2286Glnfs*21	ND	ND		437	FFPE	Pos.	4493	8490	52.92	SM
61	49	M	*NF1*	c.7199A>G	p.His2400Arg	ND	ND		394	FFPE	Pos.	1274	3909	32.59	SM
62	50	M	*NF1*	c.7909C>T	p.Arg2637*	ND	ND	Previously described in Toledo (2015)	431	FFPE	Pos.	890	1770	50.28	SM
63	51	M	*NF1*	c.3974G>T	p.Arg1325Met	ND	ND, but c.3974G>C reported as pathogenic in Netherlands: Rotterdam and France: Paris		382	FFPE	Pos.	807	1622	49.75	SM
		VUS	*MAX*	c.425C>T	p.Ser142Leu	2 of 121410 allele count. 0 homozygotes. 0.00001647 allele frequency.	ND	Previously described in Comino (2015) as nonpathogenic				67	126	53.17	GM
64	52	M	*NF1*	c.6350delC	p.Arg2119Glufs*31	ND	ND		166	Frozen	Pos.	404	851	47.5	SM
		VUS	*MEN1*	c.-10G>A		ND	ND	UMD-MEN1: Reported 3 times: likely neutral				115	368	31.3	GM (no LOH)
65	53	M	*RET*	c.2326T>C	p.Phe776Leu	ND	ND	Previously reported in Niederle (2014) in MTC	283	Blood	ND	650	1240	52.8	GM
66	54	M	*RET*	c.2647G>T	p.Ala883Ser	ND	ND	Previously reported in Gimm O (1997)	354	Frozen	ND	136	427	31.9	? (probably SM)
			RET	c.2648C>T	p.Ala883Val	ND	ND	Previously reported in Gimm O (1997)				137	427	32.2	
67	55	M	*RET*	c.2753T>C	p.Met918Thr	ND	ND	ARUP: First reference: Hofstra (1994)	116	Frozen	Pos.	638	1629	39.4	SM
68		M	*RET*	c.2753T>C	p.Met918Thr	ND	ND	ARUP: First reference: Hofstra (1994)	138	Frozen	Pos.	188	662	28.4	SM
69		M	*RET*	c.2753T>C	p.Met918Thr	ND	ND	ARUP: First reference: Hofstra (1994)	164	Frozen	Pos.	178	832	21.4	SM
70		M	*RET*	c.2753T>C	p.Met918Thr	ND	ND	ARUP: First reference: Hofstra (1994)	340	Frozen	Pos.	255	622	41.2	SM
71		M	*RET*	c.2753T>C	p.Met918Thr	ND	ND	ARUP: First reference: Hofstra (1994)	369	Frozen	ND	553	1378	40.4	? (probably SM)
72		M	*RET*	c.2753T>C	p.Met918Thr	ND	ND	ARUP: First reference: Hofstra (1994)	370	Frozen	ND	247	649	38.1	? (probably SM)

73		M	*RET*	c.2753T>C	p.Met918Thr	ND	ND	ARUP: First reference Hofstra (1994)	393	FFPE	Pos.	1454	3580	40.61	SM
74		VUS	*EPAS1*	c.1199T>C	p.Leu400Pro	ND	ND		275	Frozen	Pos.	179	353	50.9	? (probably SM)
	56	M	*EPAS1*	c.1591C>A	p.Pro531Thr	ND	ND	Previously reported Toledo (2013) as pathogenic				253	1040	24.4	
75	57	M	*EPAS1*	c.1592C>T	p.Pro531Leu	ND	ND	Previously reported Comino (2013)	154	Frozen	Pos.	211	555	38	SM
76		VUS	*EPAS1*	c.1611G>C	p.Gly537Gly	ND	ND		322	Frozen	ND	323	934	34.6	SM
	58	M	*EPAS1*	c.1615G>C	p.Asp539His	ND	ND	ND. but reported p.Asp539Tyr in Comino (2013)				323	934	34.7	
77	59	M	*SDHAF2*	c.232G>A	p.Gly78Arg	ND	Reported 2 times: Dutch Founder mutation. Spanish recurrent mutation	Previously reported in Hao (2009) as pathogenic	145	Frozen	Neg.	491	822	60	GM
78	60	M	*MAX*	c.1A>G	p.Met1Val	ND	Reported 2 times in Spain	Previously reported in Comino (2011)	191	Blood	Pos.	61	103	60.4	GM
79	61	M	*SDHD*	c.49C>T	p.Arg17*	ND	Reported 2 times	Previously reported in Neumann (2009)	147	Frozen	Neg.	176	331	53.2	GM
80	62	M	*SDHD*	c.169+5G>T		ND	ND, but reported 1 time c.169+5G>A. Netherlands. France. Splicesite mutation? cDNA: SDHD exon 2 skipping	Previously described Burnichon (2009)	336	Frozen	ND	461	574	80.5	? (probably GM)
81	63	M	*SDHD*	c.239T>G	p.Leu80Arg	ND	ND	Previously described Burnichon (2009)	328	Frozen	ND	463	888	52.4	GM
82	64	M	*SDHD*	c.334_337delACTG	p.Asp113Metfs*21	ND	ND	Previously described Burnichon (2009)	50	Blood	Neg.	439	806	54.5	GM
83	65	M	*SDHD*	c.443G>A	p.Gly148Asp	ND	Reported 1 time in France	Previously described Burnichon (2009)	296	Blood	Neg.	198	1055	18.8	GM
			SDHD	c.443G>A	p.Gly148Asp	ND	Reported 1 time in France	Previously described Burnichon (2009)		Saliva		201	1042	19.3	
84	66	M	*SDHC*	c.43C>T	p.Arg15*	ND	Reported 2 times as pathogenic	Previously described Burnichon (2009)	67	Blood	ND	729	1504	48.5	GM
85	67	M	*SDHC*	c.214C>T	p.Arg72Cys	1 (East Asian) of 121412 allele count. 0 homozygotes. 0.000008236 allele frequency.	Reported 2 times as probably pathogenic: functional domain. conserved residue. 0/164 controls	Previously described Burnichon (2009)	106	Blood	Neg.	839	1635	51.4	GM
86	68	M	*SDHC*	c.379C>T	p.His127Tyr	ND	ND	Previously reported in Buffet (2012)	141	Frozen	Neg.	492	1062	46.3	GM

87	69	M	*FH*	c.1431_1433dupAAA	p.Lys477dup	110 of 121266 allele count. 0 homozygous. 0.0009071.	Reported 9 times	Previously described in Coughlin (1998)	114	Blood	Pos.	193	497	40	GM							
88	70	M	*FH*	c.580G>A	p.Ala194Thr	10 of 120820 allel count. 0 homozygotes. 0.00008277 allele frequency	ND	Previously described in Castro (2014)	358	Frozen	ND	736	1314	56.1	GM							
89	71	M	*FH*	c.555+1G>A		ND	Reported 2 times as probably pathogenic	Previously described in Gardie (2011) in HLRCC	247	Blood	Pos.	745	1426	52.3	GM							

VUS	VUS[u]	Gene	cDNA	Protein	Sift	PolyPhen	dbSNP ID	COSMIC ID	AF	AF Amr	AF Asn	AF Af	AF Eur	LOVD	Pubmed	ExAC database	ID ALL	Sample	Alt Read Depth	Read Depth	Alt Var. Freq	Type VUS	Additional studies	Final decision
1	1	*SDHA*	c.125G>A	p.Arg42Lys	tol	benign			0	0	0	0	0	ND		1 (Latino) of 121410 allele count. 0 homozygotes. 0.000008237 allele frequency.	310	Blood	412	885	47.1	G	Negative SDHB-IHC; Positive SDHA-IHC.	Probably non pathogenic
																		FFPE	446	1058	42.16			
2	2	*SDHA*	c.155C>T	p.Ser52Phe	del.	possibly_damaging			0	0	0	0	0	ND		15 of 121302 allele count. 0 homozygotes. 0.0001237 allele frequency.	165	Frozen	89	160	55.6	G	Positive SDHB-IHC	Probably non pathogenic
3	3	*SDHA*	c.354C>T	p.Asn118Asn										ND		ND	362	Frozen	530	1136	46.9	Tumor. no blood	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic
4	4	*SDHA*	c.456+6G>T				rs371735891							ND		34 of 119486 allele count. 0 homozygotes. 0.0002846 allele frequency.	361	Blood	735	1433	51.7	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic
5	5	*SDHA*	c.723C>T	p.Asp241Asp			rs146653693		0	0	0	0	0	ND		59 of 121404 allele count. 0 homozygotes. 0.0004860 allele frequency.	253	Blood	1833	3596	51.2	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic

6	6	*SDHA*	c.770+12A>G				rs201245536		0	0	0	0	0	ND		5 of 120462 allele count. 0 homozygotes. 0.00004151 allele frequency.	65	Frozen	850	1552	54.9	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic
7	7	*SDHA*	c.1432+16A>G						0	0	0	0	0	ND		2 of 121330 allele count. 0 homozygotes. 0.00001648 alelel frequency.	90	Blood	234	429	54.8	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic
8	8	*SDHA*	c.1456C>A	p.Pro486Thr	tol.	benign	rs138190937		0	0	0	0	0	ND		ND	89	Blood	489	894	54.8	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic
9	9	*SDHA*	c.1644C>T	p.His548His			rs1126427		0	0	0	0	0	ND		2 (European non-Finnish) of 121394 allele count. 0 homozygotes. 0.00001648 allele frequency.	192	Blood	550	1200	45.9	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic
																		Frozen	613	1125	54.7			
10	10	*KIF1B*	c.146C>A	p.Ser49Tyr	del	possibly_damaging	rs143654307	COSM3470408.COSM3470409						ND	ND	10 of 121406 allele count. 0 homozygotes. 0.00008237.	170	Blood	1358	2505	54.3	G		Unknown
11	11	*KIF1B*	c.635A>C	p.Glu212Ala	tol.	probably_damaging			0	0	0	0	0	ND	ND	ND	110	Blood	932	1845	50.7	G		Unknown
12	12	*KIF1B*	c.1456C>G	p.Pro486Ala	del.	possibly_damaging	rs201500946		0.05	0	0	0	0.13	ND	ND	ND	26	Frozen	719	1365	52.8	G		Unknown
13																	86	Frozen	508	1624	31.4	G	Positive SDHB-IHC	Unknown
14																	270	Blood	1016	1925	53.3	G		Unknown
15	13	*SDHB*	c.455C>T	p.Ser152Phe	del.	Benign	rs200414835		0	0	0	0	0	ND		7 (African) of 121370 allele count. 0 homozygotes. 0.00005767 allele frequency.	218	Blood	1279	2633	48.6	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably nonpathogenic (patient origin in Africa)

16	14	*SDHB*	c.221A>G	p.Asp74Gly	del.	probably_damaging			0	0	0	0	0	ND		ND	163	Blood	83	196	42.6	G	FFPE slide to perform SDHB/SDHA-IHC requested	Posible pathogenic
17	15	*NF1*	c.4118G>T	p.Cys1373Phe	del.	possibly_damaging			0	0	0	0	0	ND		ND	68	Blood	330	718	46	G	No NF1 phenotypic features	Probably non pathogenic
18	16	*NF1*	c.4430+1G>T						0	0	0	0	0	ND		ND	180	Frozen	858	1086	79.2	G	No NF1 phenotypic features	Probably non pathogenic
19	17	*NF1*	c.4796C>T	p.Ser1599Phe	del.	probably_damaging			0	0	0	0	0	ND		ND	397	FFPE	118	658	17.93	Tumor. no blood	No NF1 phenotypic features. Positive SDHB-IHC	Probably non pathogenic
20	18	*NF1*	c.5477A>G	p.His1826Arg	del.	possibly_damaging			0	0	0	0	0	ND		ND	139	Frozen	335	738	45.4	G	No NF1 phenotypic features	Probably non pathogenic
21	19	*NF1*	c.7269_7270delCA	p.His2423Glnfs*4										ND		ND	445	FFPE	249	519	47.98	G	No NF1 phenotypic features. Positive SDHB-IHC	Probably non pathogenic
22	20	*NF1*	c.7971-7C>A						0	0	0	0	0	ND		ND	287	Blood	1036	2110	49.3	G	No NF1 phenotypic features	Probably non pathogenic
23	21	*NF1*	c.7985_7986delAC	p.Asp2662Valfs*2										ND		ND	366	Frozen	758	1705	44.6	Tumor. no blood	No LOH and no phenotypic features	Probably non pathogenic
24	22	*RET*	c.1941C>T	p.Ile647Ile			rs75225191		0	0	0	0	0	ND	Previously reported in Auricchio (1999) in Hirschsprung's disease as	11 of 121202 allele count. 0 homozygotes. 0.00009076 allele frequency.	429	FFPE	271	538	50.37	G	More blood requested to perform splicing study. Positive SDHB-IHC	Posible pathogenic

															pathogenic									
25	23	*SDHC*	c.24C>T	p.His8His					0	0	0	0	0	ND	Previously reported in Bayley (2006) as a polymorphism in Duth population (1%)	ND	278	Blood	905	1911	47.4	G	FFPE slide to perform SDHB/SDHA-IHC requested	Probably non pathogenic
26	24	*EPAS1*	c.1700T>C	p.Met567Thr	tol.	benign			0	0	0	0	0	ND	Previously reported: Comino (2013): probably non pathogenic	14 of 121324 allele count. 0 homozygotes. 0.0001154 allele frequency.	405	FFPE	1020	1964	51.93	G	Positive SDHB-IHC	Probably non pathogenic
27																	450	FFPE	1587	3744	42.39	G	Positive SDHB-IHC	Probably non pathogenic
28	25	*EPAS1*	c.1675A>G	p.Thr559Ala	tol.	benign			0	0	0	0	0	ND		ND	123	Blood	Not detected in blood			S		Probably non pathogenic
																	123	FFPE	No amplified (previously detected by SS)				Positive SDHB-IHC	
29	26	*MEN1*	c.628G>A	p.Asp210Asn	del.	probably_damaging			0	0	0	0	0	ND	UMD-MEN1: ND	ND	118	Blood	967	1936	50	G	No LOH in the tumor. Positive SDHB-IHC	Probably non pathogenic
30	27	*MDH2*	c.8C>T	p.Ser3Phe	del.	unknown			0	0	0	0	0	ND		ND	137	Frozen	54	98	55.1	G	qPCR RBP1: High levels	Probably non pathogenic
31	28	*MDH2*	c.45C>T	p.Arg15Arg			rs782800852		0	0	0	0	0	ND		7 of 14064 allele count. 0 homozygotes. 0.0004977 allele frequency.	257	Blood	137	223	62	G	Tumor to perform RBP1/MDH2 qPCR requested	Unknown
32	29	*MDH2*	c.389A>G	p.Gln130Arg	tol.	benign			0	0	0	0	0	ND		ND	61	Blood	576	1207	47.8	G	Tumor to perform RBP1/	Unknown

																							MDH2 qPCR requested	
33	30	*MDH2*	c.478G>A	p.Val160Met	del.	possibly_damaging	rs138541865		0	0	0	0	0	ND		20 of 121274 allele count. 0 homozygotes. 0.0001649 allele frequency.	255	Blood	129	229	56.3	G	Negative SDHB-IHC	Probably non pathogenic
																		FFPE	135	285	47.37			
34	31	*MDH2*	c.555+8C>T				rs200420048		0.09	0.28	0	0	0.13	ND		ND	335	Frozen	117	201	58.2	Tumor. no blood	Tumor to perform RBP1/ MDH2 qPCR requested	Unknown
35	32	*MDH2*	c.999C>T	p.Phe333Phe			rs146761624		0	0	0	0	0	ND		60 of 120964 allele count. 0 homozygotes. 0.0004960.	51	Blood	418	852	49.2	G	Tumor to perform RBP1/ MDH2 qPCR requested	Unknown
36	33	*TMEM127*	c.448G>C	p.Ala150Pro	tol.	probably_damaging			0	0	0	0	0	ND		ND	179	Frozen	998	1089	92.2	Tumor. no blood		Unknown
37	34	*TMEM127*	c.267A>G	p.Thr89Thr			rs773384410		0	0	0	0	0	ND		1 of 121216 allele count. 0 homozygotes. 0.000008250.	251	Blood	Not detected (low coverage region)			G		Probably non pathogenic
38	35	*FH*	c.1237-9C>T						0	0	0	0	0	ND		6 of 119660 allele count. 0 homozygotes. 0.00005014.	294	Blood	182	609	29.9	G	Requested blood (RNA) and FFPE slide to perform 5hmC/ 2SC	Probably non pathogenic
39	36	*FH*	c.952C>A	p.His318Asn	tol.	benign			0	0	0	0	0	ND. but reported 4 times the variant :c.952C>T p.H318Yas	ND. but reported as pathogenic the variant c.952C>T (FH enzymatic	ND	400	FFPE	6901	11778	58.59	Tumor. no blood	IHC 5hmC negative. FFPE slide to perform 2SC requested. Positive	Possible pathogenic

														pathogenic	activity reduced)								SDHB-IHC.	
40	37	*FH*	c.700A>G	p.Thr234Ala	del.	probably_damaging								ND		ND	364	Frozen	640	752	85.2	Tumor. no blood	Requested blood and FFPE slide to perform 5hmC/2SC	Unknown
41	38	*FH*	c.555+4A>G						0	0	0	0	0	ND		3 of 121390 (European Non-Finnish) allele count. 0 homozygotes. 0.00002471.	312	Blood	692	1398	49.7	G	Requested blood (RNA) and FFPE slide to perform 5hmC/2SC	Probably non pathogenic
42	39	*FH*	c.-9C>T				rs200159437		0	0	0	0	0	ND	Previously reported in Castro (2015) as a SNP	1 (South Asia) of 14190 allele count. 0 homozygotes. 0.00007047.	210	Blood	39	64	60.9	G	Requested blood (RNA) and FFPE slide to perform 5hmC/2SC	Probably non pathogenic

Supplementary table S7. Variants reported by TGPs and not validated by Sanger sequencing. FFPE: formalin fixed paraffin-embedded tumor tissue; Alt: Altered; Freq: frequency; Low coverage: low coverage of the altered variant.

ID	Reason	Sample	Gene	cDNA	Protein	Alt Read Depth	Read Depth	Alt Variant Freq
229	Homopolymeric	Blood	*KIF1B*	c.1905-8A>T		137	614	23.1
23	Homopolymeric	Blood	*KIF1B*	c.1905-4C>T		116	554	21.2
105	Low coverage	Blood	*MAX*	c.296-4T>C		4	20	20
259	Low coverage	Blood	*FH*	c.1237-8A>T		69	678	10.5

ID	Reason	Sample	Gene	cDNA	Protein	Alt Read Depth	Read Depth	Alt Variant Freq
355	Low coverage	Frozen	*VHL*	c.269A>G	p.Asn90Ser	3	15	20.0
139	Low coverage	Frozen	*SDHB*	c.490C>A	p.Gln164Lys	6	60	10
338	Low coverage	Frozen	*NF1*	c.5789G>T	p.Cys1930Phe	13	115	11.4
137	Low coverage	Frozen	*SDHC*	c.158C>A	p.Ser53Tyr	10	62	16.1

ID	Reason	Sample	Gene	cDNA	Protein	Alt Read Depth	Read Depth	Alt Variant Freq
291	Low coverage	FFPE	*HRAS*	c.179G>A	p.Gly60Asp	75	1453	5.16
451	Low coverage	FFPE	*HRAS*	c.175G>A	p.Ala59Thr	78	2286	3.41
378	Low coverage	FFPE	*VHL*	c.231C>A	p.Cys77*	22	494	4.45
398	Low coverage	FFPE	*NF1*	c.227delA	p.Asn78Ilefs*7	91	2302	3.95
403	Low coverage	FFPE	*NF1*	c.2834T>C	p.Phe945Ser	56	1538	3.64
412	Low coverage	FFPE	*NF1*	c.5806delA	p.Lys1936Asnfs*6	150	4753	3.16
398	Low coverage	FFPE	*NF1*	c.6535C>T	p.Arg2179Cys	507	9128	5.55
450	Low coverage	FFPE	*NF1*	c.7195A>G	p.Arg2399Gly	41	1289	3.18
292	Low coverage	FFPE	*EPAS1*	c.1734C>T	p.Ala578Ala	95	2916	3.26
413	Low coverage	FFPE	*MAX*	c.247C>T	p.Gln83*	47	1467	3.2
395	Low coverage	FFPE	*TMEM127*	c.480_482delGCA	p.Gln160del	248	2726	9.1
420	Low coverage	FFPE	*FH*	c.1219G>A	p.Val407Ile	46	794	5.79
395	Low coverage	FFPE	*FH*	c.952C>T	p.His318Tyr	321	6534	4.91
432	Low coverage	FFPE	*FH*	c.679C>T	p.Gln227*	187	2872	6.51
421	Low coverage	FFPE	*FH*	c.578C>T	p.Thr193Ile	37	765	4.84
	Low coverage	FFPE	*FH*	c.7C>T	p.Arg3*	44	447	9.84

IX- APPENDIX II: ARTICLES RELATED TO THE THESIS

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Recommendations for somatic and germline genetic testing of single pheochromocytoma and paraganglioma based on findings from a series of 329 patients.

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ABSTRACT:

BACKGROUND: Nowadays, 65-80% of pheochromocytoma and paraganglioma (PPGL) cases are explained by germline or somatic mutations in one of 22 genes. Several genetic testing algorithms have been proposed, but they usually exclude sporadic-PPGLs (S-PPGLs) and none include somatic testing. We aimed to genetically characterise S-PPGL cases and propose an evidence-based algorithm for genetic testing, prioritising DNA source.

METHODS: The study included 329 probands fitting three criteria: single PPGL, no syndromic and no PPGL family history. Germline DNA was tested for point mutations in RET and for both point mutation and gross deletions in VHL, the SDH genes, TMEM127, MAX and FH. 99 tumours from patients negative for germline screening were available and tested for RET, VHL, HRAS, EPAS1, MAX and SDHB.

RESULTS: Germline mutations were found in 46 (14.0%) patients, being more prevalent in paragangliomas (PGLs) (28.7%) than in pheochromocytomas (PCCs) (4.5%) ($p=6.62\times10(-10)$). Somatic mutations were found in 43% of those tested, being more prevalent in PCCs (48.5%) than in PGLs (32.3%) ($p=0.13$). A quarter of S-PPGLs had a somatic mutation, regardless of age at presentation. Head and neck PGLs (HN-PGLs) and thoracic-PGLs (T-PGLs) more commonly had germline mutations ($p=2.0\times10(-4)$ and $p=0.027$, respectively). Five of the 29 metastatic cases harboured a somatic mutation, one in HRAS.

CONCLUSIONS: We recommend prioritising testing for germline mutations in patients with HN-PGLs and T-PGLs, and for somatic mutations in those with PCC. Biochemical secretion and SDHB-immunohistochemistry should guide genetic screening in abdominal-PGLs. Paediatric and metastatic cases should not be excluded from somatic screening.

J Mol Diagn.

PheoSeq: A Targeted Next-Generation Sequencing Assay for Pheochromocytoma and Paraganglioma Diagnostics. Practical Experience in 453 Patients.

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ABSTRACT:

Background: Genetic diagnosis is recommended for all pheochromocytoma and paraganglioma (PPGL) cases, as driver mutations are identified approximately 80%. As the list of related genes expands, genetic diagnosis becomes more time-consuming, and targeted next generation sequencing (NGS) has emerged as a cost-effective tool. This study aimed to optimize targeted-NGS in PPGL genetic diagnostics.

Methodology: A workflow based on 2 customized targeted-NGS assays was validated to study the 18 main PPGL genes in germline and frozen tumor DNA, being one of them specifically directed towards formalin-fixed paraffin-embedded tissue. The series involved 453 unrelated PPGL patients, of which 30 had known mutations and were used as controls. Partial screening using Sanger had been performed in 275 (WTPS). NGS results were complemented with a study of gross deletions

Results: NGS assay sensitivity was ≥99.4%, regardless of DNA source. We identified 45 variants of unknown significance and 89 pathogenic mutations, the latter being germline in 29 (7.2%) and somatic in 58 (31.7%) of the 183 tumors studied. In 13 WTPS the causal mutation could be identified.

Conclusions: We demonstrated that both assays are an efficient and accurate alternative to conventional sequencing. Their application facilitates the study of minor PPGL genes, and enables genetic diagnoses in patients with incongruent or missing clinical data, that would otherwise be missed.

IX- APPENDIX II: OTHER ARTICLES

Cancer Genet. 2016 Jun;209(6):272-7.

ATRX driver mutation in a composite malignant pheochromocytoma.

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ABSTRACT:

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are tumors arising from the adrenal medulla and sympathetic/parasympathetic paraganglia, respectively.

Approximately 40% of PCCs/PGLs are due to germline mutations in one of 16 susceptibility genes, and a further 30% are due to somatic alterations in 5 main genes. Recently, somatic ATRX mutations have been found in succinate dehydrogenase (SDH)-associated hereditary PCCs/PGLs. In the present study we applied whole-exome sequencing to the germline and tumor DNA of a patient with metastatic composite PCC and no alterations in known PCC/PGL susceptibility genes. A somatic loss-of-function mutation affecting ATRX was identified in tumor DNA. Transcriptional profiling analysis classified the tumor within cluster 2 of PCCs/PGLs (without SDH gene mutations) and identified downregulation of genes involved in neuronal development and homeostasis (NLGN4, CD99 and CSF2RA) as well as upregulation of Drosha, an important gene involved in miRNA and Rrna processing. CpG island methylator phenotype typical of SDH gene-mutated tumors was ruled out, and SNP array data revealed a unique profile of gains and losses.

Finally, we demonstrated the presence of alternative lengthening of telomeres in the tumor, probably associated with the failure of ATRX functions. In conclusion, somatic variants affecting ATRX may play a driver role in sporadic PCC/PGL.

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Functional and in silico assessment of MAX variants of unknown significance.

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ABSTRACT:

The presence of germline mutations affecting the MYC-associated protein X (MAX) gene has recently been identified as one of the now 11 major genetic predisposition factors for the development of hereditary pheochromocytoma and/or paraganglioma. Little is known regarding how missense variants of unknown significance (VUS) in MAX affect its pivotal role in the regulation of the MYC/MAX/MXD axis. In the present study, we propose a consensus computational prediction based on five "state-of-the-art" algorithms. We also describe a PC12-based functional assay to assess the effects that 12 MAX VUS may have on MYC's E-box transcriptional activation. For all but two of these 12 VUS, the functional assay and the consensus computational prediction gave consistent results; we classified seven variants as pathogenic and three as nonpathogenic.

The introduction of wild-type MAX cDNA into PC12 cells significantly decreased MYC's ability to bind to canonical E-boxes, while pathogenic MAX proteins were not able to fully repress MYC activity. Further clinical and molecular evaluation of variant carriers corroborated the results obtained with our functional assessment. In the absence of clear heritability, clinical information, and molecular data, consensus computational predictions and functional models are able to correctly classify VUS affecting MAX.KEY

MESSAGES: A functional assay assesses the effects of MAX VUS over MYC transcriptional activity. A consensus computational prediction and the functional assay show high concordance. Variant carriers' clinical and molecular data support the functional assessment.

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DNA Methylation Profiling in Pheochromocytoma and Paraganglioma Reveals Diagnostic and Prognostic Markers.

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ABSTRACT:

PURPOSE: Pheochromocytoma and paraganglioma (PPGL) are rare neuroendocrine tumors, associated with highly variable postoperative evolution. The scarcity of reliable PPGL prognostic markers continues to complicate patient management. In this study, we explored genome-wide DNA methylation patterns in the context of PPGL malignancy to identify novel prognostic markers.

EXPERIMENTAL DESIGN: We retrospectively investigated DNA methylation patterns in PPGL with and without metastases using high-throughput DNA methylation profiling data (Illumina 27K)

from two large, well-characterized discovery (n = 123; 24 metastatic) and primary validation (n = 154; 24 metastatic) series. Additional validation of candidate CpGs was performed by bisulfite pyrosequencing in a second independent set of 33 paraffin-embedded PPGLs (19 metastatic).

RESULTS: Of the initial 86 candidate CpGs, we successfully replicated 52 (47 genes), associated with metastatic PPGL. Of these, 48 CpGs showed significant associations with time to progression even after correcting for SDHB genotype, suggesting their value as prognostic markers independent of genetic background. Hypermethylation of RDBP (negative elongation factor complex member E) in metastatic tumors was further validated by bisulfite pyrosequencing [Δβmetastatic-benign = 0.29, P = 0.003; HR, 1.4; 95% confidence interval (CI), 1.1-2.0; P = 0.018] and may alter transcriptional networks involving (RERG, GPX3, and PDZK1) apoptosis, invasion, and maintenance of DNA integrity.

CONCLUSIONS: This is the first large-scale study of DNA methylation in metastatic PPGL that identifies and validates prognostic markers, which could be used for stratifying patients according to risk of developing metastasis. Of the three CpGs selected for further validation, one (RDBP) was clearly confirmed and could be used for stratifying patients according to the risk of developing metastases.

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SDHB/SDHA immunohistochemistry in pheochromocytomas and paragangliomas: a multicenter interobserver variation analysis using virtual microscopy: a Multinational Study of the European Network for the Study of Adrenal Tumors (ENS@T).

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ABSTRACT:

Despite the established role of SDHB/SDHA immunohistochemistry as a valuable tool to identify patients at risk for familial succinate dehydrogenase-related pheochromocytoma /paraganglioma syndromes, the reproducibility of the assessment methods has not as yet been determined. The aim of this study was to investigate interobserver variability among seven expert endocrine pathologists using a web-based virtual microscopy approach in a large multicenter pheochromocytoma/paraganglioma cohort (n=351): (1) 73 SDH mutated, (2) 105 non-SDH mutated, (3) 128 samples without identified SDH-x mutations, and (4) 45 with incomplete SDH molecular genetic analysis. Substantial agreement among all the reviewers was observed either with a two-tiered classification (SDHB κ=0.7338; SDHA κ=0.6707) or a three-tiered classification approach (SDHB κ=0.6543; SDHA κ=0.7516). Consensus was achieved in 315 cases (89.74%) for SDHB immunohistochemistry and in 348 cases (99.15%) for SDHA immunohistochemistry. Among the concordant cases, 62 of 69 (~90%) SDHB-/C-/D-/AF2-mutated cases displayed SDHB immunonegativity and SDHA immunopositivity, 3 of 4 (75%) with SDHA mutations showed loss of SDHA/SDHB protein expression, whereas 98 of 105 (93%) non-SDH-x-mutated counterparts demonstrated retention of SDHA/SDHB protein expression. Two SDHD-mutated extra-adrenal paragangliomas were scored as SDHB immunopositive, whereas 9 of 128 (7%) tumors without identified SDH-x mutations, 6 of 37 (~16%) VHL-mutated, as well as 1 of 21 (~5%) NF1-mutated tumors were evaluated as SDHB immunonegative. Although 14 out of those 16 SDHB-immunonegative cases were nonmetastatic, an overall significant correlation between SDHB immunonegativity and malignancy was observed (P=0.00019). We conclude that SDHB/SDHA immunohistochemistry is a reliable tool to identify patients with SDH-x mutations with an additional value in the assessment of genetic variants of unknown significance. If SDH molecular genetic analysis fails to detect a mutation in SDHB-immunonegative tumor, SDHC promoter methylation and/or VHL/NF1 testing with the use of targeted next-generation sequencing is advisable.

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Whole-exome sequencing identifies MDH2 as a new familial paraganglioma gene.

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ABSTRACT: Disruption of the Krebs cycle is a hallmark of cancer. IDH1 and IDH2 mutations are found in many neoplasms, and germline alterations in SDH genes and FH predispose to pheochromocytoma/paraganglioma and other cancers. We describe a paraganglioma family carrying a germline mutation in MDH2, which encodes a Krebs cycle enzyme. Whole-exome sequencing was applied to tumor DNA obtained from a man age 55 years diagnosed with multiple malignant paragangliomas. Data were analyzed with the two-sided Student's t and

Mann-Whitney U tests with Bonferroni correction for multiple comparisons. Between six- and 14-fold lower levels of MDH2 expression were observed in MDH2-mutated tumors compared with control patients. Knockdown (KD) of MDH2 in HeLa cells by shRNA triggered the accumulation of both malate (mean ± SD: wild-type [WT] = 1±0.18; KD = 2.24±0.17, P = .043) and fumarate (WT = 1±0.06; KD = 2.6±0.25, P = .033), which was reversed by transient introduction of WT MDH2 cDNA. Segregation of the mutation with disease and absence of MDH2 in mutated tumors revealed MDH2 as a novel pheochromocytoma/paraganglioma susceptibility gene.J Mol Med (Berl). 2015 Nov;93(11):1247-55.

Hum Mol Genet. 2014 May 1;23(9):2440-6.

Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas.

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ABSTRACT:

Malignant pheochromocytoma (PCC) and paraganglioma (PGL) are mostly caused by germline mutations of SDHB, encoding a subunit of succinate dehydrogenase. Using whole-exome sequencing, we recently identified a mutation in the FH gene encoding fumarate hydratase, in a PCC with an 'SDH-like' molecular phenotype. Here, we investigated the role of FH in PCC/PGL predisposition, by screening for germline FH mutations in a large international cohort of patients. We screened 598 patients with PCC/PGL without mutations in known PCC/PGL susceptibility genes. We searched for FH germline mutations and large deletions, by direct sequencing and multiplex ligation-dependent probe amplification methods. Global alterations in DNA methylation and protein succination were assessed by immunohistochemical staining for 5-hydroxymethylcytosine (5-hmC) and S-(2-succinyl) cysteine (2SC), respectively. We identified five pathogenic germline FH mutations (four missense and one splice mutation) in five patients. Somatic inactivation of the second allele, resulting in a loss of fumarate hydratase activity, was demonstrated in tumors with FH mutations. Low tumor levels of 5-hmC, resembling those in SDHB-deficient tumors, and positive 2SC staining were detected in tumors with FH mutations. Clinically, metastatic phenotype (P = 0.007) and multiple tumors (P = 0.02) were significantly more frequent in patients with FH mutations than those without such mutations. This study reveals a new role for FH in susceptibility to malignant and/or multiple PCC/PGL. Remarkably, FH-deficient PCC/PGLs display the same pattern of epigenetic deregulation as SDHB-mutated malignant PCC/PGL.

Therefore, we propose that mutation screening for FH should be included in PCC/PGL genetic testing, at least for tumors with malignant behavior.

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Targeted Sequencing Reveals Low-Frequency Variants in EPHA Genes as Markers of Paclitaxel-Induced Peripheral Neuropathy.

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ABSTRACT:

PURPOSE: Neuropathy is the dose-limiting toxicity of paclitaxel and a major cause for decreased quality of life. Genetic factors have been shown to contribute to paclitaxel neuropathy susceptibility; however, the major causes for interindividual differences remain unexplained. In this study, we identified genetic markers associated with paclitaxel-induced neuropathy through massive sequencing of candidate genes.

EXPERIMENTAL DESIGN: We sequenced the coding region of 4 EPHA genes, 5 genes involved in paclitaxel pharmacokinetics, and 30 Charcot-Marie-Tooth genes, in 228 cancer patients with no/low neuropathy or high-grade neuropathy during paclitaxel treatment. An independent validation series included 202 paclitaxel-treated patients. Variation-/gene-based analyses were used to compare variant frequencies among neuropathy groups, and Cox regression models were used to analyze neuropathy along treatment.

RESULTS: Gene-based analysis identified EPHA6 as the gene most significantly associated with paclitaxel-induced neuropathy. Low-frequency nonsynonymous variants in EPHA6 were present exclusively in patients with high neuropathy, and all affected the ligand-binding domain of the protein. Accumulated dose analysis in the discovery series showed a significantly higher neuropathy risk for EPHA5/6/8 low-frequency nonsynonymous variant carriers [HR, 14.60; 95% confidence interval (CI), 2.33-91.62; P = 0.0042], and an independent cohort confirmed an increased neuropathy risk (HR, 2.07; 95% CI, 1.14-3.77; P = 0.017). Combining the series gave an estimated 2.5-fold higher risk of neuropathy (95% CI, 1.46-4.31; P = 9.1 × 10(-4)).

CONCLUSIONS: This first study sequencing EPHA genes revealed that low-frequency variants in EPHA6, EPHA5, and EPHA8 contribute to the susceptibility to paclitaxel-induced neuropathy. Furthermore, EPHA's neuronal injury repair function suggests that these genes might constitute important neuropathy markers for many neurotoxic drugs. Clin Cancer Res; 1-9. ©2016 AACR.

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High frequency and founder effect of the CYP3A4*20 loss-of-function allele in the Spanish population classifies CYP3A4 as a polymorphic enzyme.

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ABSTRACT:

Cytochrome P450 3A4 (CYP3A4) is a key drug-metabolizing enzyme. Loss-of-function variants have been reported as rare events, and the first demonstration of a CYP3A4 protein lacking functional activity is caused by CYP3A4*20 allele. Here we characterized the world distribution and origin of CYP3A4*20 mutation. CYP3A4*20 was determined in more than 4000 individuals representing different populations, and haplotype analysis was performed using CYP3A polymorphisms and microsatellite markers. CYP3A4*20 allele was present in 1.2% of the Spanish population (up to 3.8% in specific regions), and all CYP3A4*20 carriers had a common haplotype.

This is compatible with a Spanish founder effect and classifies CYP3A4 as a polymorphic enzyme. This constitutes the first description of a CYP3A4 loss-of-function variant with high frequency in a population. CYP3A4*20 results together with the key role of CYP3A4 in drug metabolism support screening for rare CYP3A4 functional alleles among subjects with adverse drug events in certain populations.

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VEGF, VEGFR3, and PDGFRB protein expression is influenced by RAS mutations in medullary thyroid carcinoma.

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ABSTRACT:

BACKGROUND: Tyrosine kinase inhibitors (TKIs) have achieved remarkable clinical results in medullary thyroid carcinoma (MTC) patients. However, the considerable variability in patient response to treatment with TKIs remains largely unexplained. There is evidence that it could be due, at least in part, to alterations in genes associated with the disease via their effect on the expression of TKI targets. The objective of this study was to evaluate the influence of RAS mutations on the expression levels in MTC tumors of eight key TKI target proteins.

METHODS: We assessed by immunohistochemistry the expression of EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3 in a series of 84 primary MTC tumors that had previously been molecularly characterized, including 14 RAS-positive, 18 RET(M918T)-positive, and 24 RET(C634)-positive tumors, as well as 15 wild-type tumors with no mutations in the RET or RAS genes.

RESULTS: In contrast to RET-positive tumors, RAS-positive tumors expressed neither PDGFRB nor MET (p=0.0060 and 0.047, respectively). Similarly, fewer RAS-positive than RET-related tumors expressed VEGFR3 (p=0.00062). Finally, wild-type tumors expressed VEGF more often than both RAS- and RET-positive tumors (p=0.0082 and 0.011, respectively).

CONCLUSIONS: This is the first study identifying that the expression of TKI targets differs according to the presence of RAS mutations in MTC. This information could potentially be used to select the most beneficial TKI treatment for these patients.

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DNA methylation profiling of well-differentiated thyroid cancer uncovers markers of recurrence free survival.

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ABSTRACT:

Thyroid cancer is a heterogeneous disease with several subtypes characterized by cytological, histological and genetic alterations, but the involvement of epigenetics is not well understood. Here, we investigated the role of aberrant DNA methylation in the development of well-differentiated thyroid tumors. We performed genome-wide DNA methylation profiling in the largest well-differentiated thyroid tumor series reported to date, comprising 83 primary tumors as well as 8 samples of adjacent normal tissue. The epigenetic profiles were closely related to not only tumor histology but also the underlying driver mutation; we found that follicular tumors had higher levels of methylation, which seemed to accumulate in a progressive manner along the tumorigenic process from adenomas to carcinomas. Furthermore, tumors harboring a BRAF or RAS mutation had a larger number of hypo- or hypermethylation events, respectively. The aberrant methylation of several candidate genes potentially related to thyroid carcinogenesis was validated in an independent series of 52 samples.

Furthermore, through the integration of methylation and transcriptional expression data, we identified genes whose expression is associated with the methylation status of their promoters. Finally, by integrating clinical follow-up information with methylation levels we propose etoposide-induced 2.4 and Wilms tumor 1 as novel prognostic markers related to recurrence-free survival. This comprehensive study provides insights into the role of DNA methylation in well-differentiated thyroid cancer development and identifies novel markers associated with recurrence-free survival.